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AUTORADIOGRAPHIC STUDIES ON MOSQUITOES AND
FILARIAL PARASITES ^{IN} OF MOSQUITOES

A thesis submitted for the Degree of Doctor
of Philosophy in the Faculty of Science of
the University of London

by

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ABSTRACT

Aspects of the host parasite relationships which exist between larvae of the filarial nematode Brugia patei and two mosquito intermediate hosts are explored with the aid of an autoradiographic technique and selected histochemical staining methods.

In Aedes togoi normal development of the worm occurs following a blood meal taken from a domestic cat infected with microfilariae of Brugia patei. Conversely Anopheles labranchiae atroparvus is refractory to infection with Brugia patei and it has been established previously that in the latter mosquito the parasite exhibits a number of abnormalities in its pattern of growth, and it rarely develops successfully to the second larval stage. The present study therefore concentrates largely on the principal histological events which take place in both the parasite and its two mosquito hosts during this early critical period of development.

The results describe the incorporation of [H_3]-labelled nucleosides, amino acids and carbohydrates in normal and abnormal worms and include autoradiographic and histochemical features of the host reaction elicited by Brugia patei when parasitic in Anopheles labranchiae atroparvus.

Autoradiographic observations are also recorded for both host mosquitoes. Particular emphasis is given to the alimentary canal, fat body, heart, pericardial cells and female reproductive tract during the period when the mosquito is parasitised by the nematode. Differences between parasitised individuals and uninfected controls are noted.

These findings are discussed in relation to our general understanding of host parasite relationships in insects and entomophilic nematodes.

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PART 1GENERAL INTRODUCTION

Nematodes of the superfamily Filarioidea (Chabaud and Anderson, 1959) are parasites of the lymphatics and body cavities of vertebrates. The first stage larvae (microfilariae) are released from the uterus of the adult female and enter the host blood stream. Transport to a fresh host is effected when the blood infected with microfilariae is ingested by a blood feeding (haematophagous) arthropod (Hawking and Worms, 1961); see also Gooding, 1972). Providing the latter is a susceptible host, the microfilariae undergo a phase of growth and differentiation, including two moults, a process which results in their transformation into third stage larvae (Feng, 1936; Lavoipierre, 1958; Taylor, 1960; Laurence and Pester, 1961; Schacher, 1962a; Jayewardene, 1963; Orihel, 1967; Schacher and Khalil, 1968).

At this point further development is arrested and metamorphosis to a young adult is deferred pending the successful transport of the third stage larvae into a vertebrate host. An opportunity for this arises when the parasitised arthropod takes a further meal of blood. The infective larvae enter the puncture wound that remains in the vertebrate skin following the withdrawal of the biting mouthparts of the arthropod (McGreevy et al, 1974). Inside the vertebrate (definitive) host the third stage larvae resume development and if this is successful will progress through a further two moults, become sexually mature, eventually producing a patent infection (Schacher, 1962b; Ash and Riley, 1970a; 1970b; Ash and Schacher, 1971).

It is essential for the completion of the life cycle that the nematode is ingested by a susceptible intermediate host. In the "wrong host" the well ordered sequence of mitotic cell divisions and growth which would eventually transform the first stage larva into the third stage form, does not normally occur. Instead, growth is stunted, and a series of asynchronous cell divisions often produce a series of bizarre larvae which show developmental abnormalities of the gut (Laurence, 1970; Oothuman, Simpson and Laurence, 1974), these abnormal events occurring within 48 hours of the entry of the larvae into the non-susceptible (refractory) host.

In the mosquito Aedes aegypti L. resistance and susceptibility to infection by the filarial nematodes Brugia malayi Brug and Dirofilaria immitis is known to have a genetic basis, susceptibility being controlled by a single sex-linked gene (MacDonald, 1962; McGreevy, McClelland and Lavoipierre, 1974; see also Barr, 1975). However, Laurence (1970) has correctly pointed out that there appears to be no single mechanism that will prevent or promote filarial development and which is common to all mosquitoes. In fact our general understanding of the cellular relationships which exist between filarial nematodes and their mosquito intermediate hosts remains poor, in spite of the efforts of previous researchers to spotlight these deficiencies (Lavoipierre, 1958; Nelson, 1964). It is obviously desirable in the long term to relate defence mechanisms which have a simple genetic basis to their cellular expression within the arthropod. If the inheritance of refractoriness is simple it may prove possible by genetic manipulation to increase the efficiency of the arthropod defence mechanisms, with the eventual aim of controlling filarial and other arthropod borne diseases by the replacement of vector populations with those of enhanced resistance (Barr, 1975). In the case of mosquitoes, which remain the principal vectors of filarial disease (Hawking and Worms, 1961) an immediate objective is to expand our knowledge of the way in which mosquito defence mechanisms operate when challenged by metazoan parasites, including filarial larvae. Interesting results have already been obtained by workers adopting an experimental approach to the problem (Gwadz and Spielman, 1974; Andreadis, Flanagan and Kaczor, 1975; Andreadis and Hall, 1976). Even so, our knowledge of these defence mechanisms still remains largely descriptive in nature (Poinar, 1969; Laurence, 1970; Brunhes and Brunhes, 1972; Oothuman, Simpson and Laurence, 1974).

An appropriate, and previously unexploited, methodology for the study of host parasite relationships between filaria and their mosquito hosts is the technique of autoradiography (Gahan, 1972; Rogers, 1973). The latter is a tool that has been used widely by workers in disciplines that are concerned with problems related to cell metabolism and kinetics (Lamerton and Fry, 1963; Cleaver, 1967; Feindendegen, 1967).

This study has used a simple autoradiographic technique to study the normal development of the filarial nematode Brugia patee (Buckley,

Nelson and Heisch, 1958) in the mosquito Aedes togoi (Theobald), and the abnormal development of this parasite in the refractory mosquito Anopheles labranchiae atroparvus. The histology of normal development up to the first moult of Brugia patei in Aedes togoi has been described in detail by Laurence and Simpson (1971), and the histology of abnormal development of Brugia patei in Anopheles labranchiae atroparvus, by Oothuman, Simpson and Laurence (1974).

This study provides basic information on the uptake of nutrients by normal and abnormal worms, and on the nature and possible origin of the encapsulation material which arises around the parasite in the refractory host. Observations are included on the principal autoradiographic features of the host circulatory system, fat body, alimentary canal and reproductive organs, during the period of filarial infection. Where possible, the autoradiographic findings are supported by related histochemical studies.

PART 2MATERIALS AND METHODS

The mosquitoes Aedes (Finlaya) togoi Theobald and Anopheles labranchiae atroparvus Van Thiel used in these studies were obtained from laboratory colonies maintained routinely in the Department of Medical Entomology at the London School of Hygiene and Tropical Medicine.

Following eclosion the mosquitoes were kept in 25 cm cube cages, each containing pads soaked in sucrose. A humidity of above 70% was achieved in the cages by the practice of draping dampened lint over the top of each cage and enclosing the whole in a polythene cover.

Blood-fed non-parasitised females were obtained by feeding on the forearm of a human volunteer, or else on a guinea pig, anaesthetised with Nembutal. Parasitised females were obtained by feeding on the blood of domestic cats infected with either of the filarial nematodes Brugia pahangi (Buckley and Edeson, 1956) or Brugia pateri (Buckley, Nelson and Heisch, 1958). Cats infected with these worms are maintained in the Department of Medical Helminthology at the London School of Hygiene and Tropical Medicine.

The method used to infect the mosquitoes consisted of laying an infected cat, anaesthetised with Nembutal, across the top of a cage containing Aedes togoi and Anopheles labranchiae atroparvus. Engorged females of each species were maintained in an incubator at 26°C. A damp lint was used as before to provide humidity and the mosquitoes continued to have access to pads soaked in sucrose. On average the females used for blood feeding were aged one week following eclosion.

1. Preparation of mosquitoes for paraffin wax processing

(a) Routine methods

Unless stated otherwise, mosquitoes of both species were fixed at the following physiological conditions post-emergence.

Newly emerged adults (0-24 hours).

Unfed adults (2-5 days).

Bloodfed adults at 24, 48, 72, 96 hours and at
5, 6, 7, 8, 10 and 14 days post blood meal.

Both uninfected and filarially infected mosquitoes were used. Fixation was begun by plunging the mosquitoes directly into a petri dish containing fixative where the wings and legs were removed. The insects were then transferred to 3 x 1 inch screw capped tubes of fresh fixative. Occasionally mosquitoes were fixed directly without the prior removal of wings and legs, and in such instances the wings and legs were removed after the insects had been transferred to 70% ethanol, or to whatever fluid succeeded the fixation step.

The following fixatives were employed :-

FA (formalin alcohol, one volume 40% formaldehyde: nine volumes 100% ethanol), Carnoy (Lillie, 1965), TCA (1% trichloroacetic acid in 80% ethanol) and BD (Duboscq brazil). The formula for the latter fixative is given in Gurr (1958). Fixation was begun at 4°C but was continued at room temperature (21-23°C).

Despite the existence of a wide literature on the fixation of animal tissues (see the reviews of Pearse, 1968; Hopwood, 1969; Williams, 1969; Stoward, 1973) no satisfactory criteria exist which will unequivocally determine the point at which fixation of a tissue is complete. The whole process remains in an enigmatic state. The procedure adopted here was to work with material that had been immersed in fixative for extended periods. The usual practice was to fix for 48 hours in FA, BD or TCA although no histological features that could be attributed to differences in the duration of fixation were recognised in sections of mosquitoes that had been fixed in FA for either 48 hours or for one month. According to Gurr (1963) tissues can be stored in BD. Mosquitoes were fixed in Carnoy for 18-24 hours at 0-4°C to avoid excessive tissue shrinkage that would result from fixation at room temperature.

Following fixation in FA, TCA and Carnoy, mosquitoes were transferred into 70% ethanol and stored overnight. Dehydration was completed by a one hour immersion in 90% ethanol, followed by two changes in Absolute ethanol of one hour in each change. The insects were placed in cedar wood oil overnight, then given a second change of fresh oil and if not kept for storage, were on the third day transferred to chloroform (two 30 minute changes) and impregnated with paraplast at 58-60°C. Three changes of wax were given and the final impregnation was carried out under vacuum (20 mm Hg). The time spent in the wax was within the range 3-6 hours.

Following fixation in BD, material was dehydrated through three changes of supercedrol, with one day spent in each change and after clearing in chloroform, embedding in paraplast was carried out as described above.

(b) Freeze drying

As an alternative to fixation mosquitoes were subjected to freeze drying (reviewed by Pearse, 1968), a process whereby tissue is frozen rapidly at a temperature until all but a tightly bound water fraction remains. This rapid freezing (quenching) results in water in the tissues being converted to ice without excessive formation of ice crystals. The frozen and dried tissue is then brought to room temperature and embedded directly in paraffin wax or fixed by a liquid but anhydrous fixative or by hot fixative vapour.

Mosquitoes were either anaesthetised for 1-2 minutes in the cold drawer of a refrigerator or otherwise immobilised by stunning. The latter was achieved by a gentle tapping of the collecting tube containing the insects. Anaesthetised mosquitoes were tipped straight into the quenching agent which was contained in a thick walled test tube or beaker suspended in a 1 litre Dewar vessel of liquid nitrogen. Of the two quenching agents tried iso-pentane was preferred to dichloro-difluoromethane (Arcton 12) as the mosquitoes floated on the latter. Mosquitoes were quenched for a minimum period of 15 seconds before being transferred swiftly to the drying plattern of a Pearse-Edward tissue drier with a plattern temperature set to -70 to -80°C. Forceps

prechilled by dipping into liquid nitrogen were used to effect the transfer from the holder to the plattern. The insects were dried under vacuum for 18-24 hours. The freeze dried insects were then allowed to return to room temperature before being transferred to a gauze strip resting on a layer of paraformaldehyde contained in a rectangular jar. The latter was then placed in an oven set at 60°C. The formaldehyde vapour generated was allowed to act upon the tissue for 18-24 hours. The resulting freeze dried vapour fixed (FDVF) material was then embedded in paraplast as described previously. Although it was possible to cut sections of mosquitoes that had been freeze dried and embedded directly into paraplast without fixation, the sections did not survive any type of treatment administered to them afterwards.

2. Sectioning of mosquitoes

(a) Paraplast

Whether for the purpose of routine histology, histochemical study or for subsequent autoradiographic treatment, individual mosquitoes were sectioned serially on a Spencer 820 rotary microtome set at micrometer reading of 5 μ m with a knife angle of 3° for hand sharpened knives and an angle of 5° for knives sharpened on a Shandon Elliot automatic knife sharpener.

Sections were floated out on distilled water that was covering 3 x 1 glass slides subbed with glycerin albumin. The expanded sections were allowed to dry overnight in a 37°C oven before being considered for staining.

(b) Cryostat

Mosquitoes were stunned or anaesthetised and following the removal of the wings and legs, were quenched in the manner described previously for freeze drying. The quenched tissue was transferred to a microtome block holder with Tissue-tek (Ames Co) and then quickly transferred to a Pearse-Slee cryostat with a cabinet temperature of -20°C and a micrometer feed setting of 8 μ m. In order to obtain smooth, uncrumpled

sections, the cutting speed had to be extremely slow. This could only be done with the manual feed. Serial ribbons of up to 5 sections were collected on uncoated 22 x 22 mm cover glasses and allowed to dry. Sections that were not fixed soon after drying were stored in the cold cabinet of the cryostat until required. Unfixed sections were not kept for longer than 48 hours. Ice cold 10% formalin was used as a general purpose fixative, sections being fixed for either 5-10 minutes, 1 hour, or overnight (18-24 hours).

Females of uninfected Aedes togoi and Anopheles labranchiae atroparvus were prepared for cryostat sectioning at the following physiological conditions post eclosion :- newly emerged (<24 hours), unfed (2-5 days), blood fed (24, 48, 72, 96 hours pbm). Females of Anopheles labranchiae atroparvus infected with Brugia patei were sectioned at 9 days pbm.

3. Preparation of mosquitoes for resin embedding and sectioning

A disadvantage of paraffin wax type processing methods is the varying degree of tissue shrinkage and distortion, accompanied by the removal of triglycerides and other lipids which follow the use of these methods. Here, use was made of a resin embedding technique to provide thin 1-2 μ m. sections of various mosquito tissues from uninfected individuals of Anopheles labranchiae atroparvus and Aedes togoi. As this material is being used for basic ultrastructural studies (not included in this study), mosquitoes were treated in order to meet the more exacting conditions demanded by electron microscopy. These thin sections proved valuable when assessing the extent of tissue artifacts present in routinely prepared paraplast sections.

Adult female mosquitoes at various physiological conditions post eclosion were stunned and the wings and legs quickly removed. Each individual was separated into head, thorax and abdomen (the tip of the abdomen was cut off) and all these parts placed into ice cold fixative, using either 2.5% or 4% glutaraldehyde in 0.1M S-collidine buffer (pH 7.4) or in 4% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) for periods of up to 24 hours. Fixation, although begun ice cold, was continued at 4°C. Tissues were given several changes of buffer and then stored overnight in the third buffer change. Tissues were post fixed in 1 or 2% Osmium

tetroxide in 0.1M S-collidine buffer (pH 7.4) for 2 hours at 4°C, rinsed thoroughly in buffer, dehydrated through a graded ethanol series and embedded in Spurr resin. To ensure adequate penetration of the latter all tissues were soaked in resin diluted with absolute ethanol. The tissues were left overnight in pure resin at room temperature, before polymerising at 60°C.

Sections 1-2 µm thick, were cut on a model 18000 LKB pyramitome, or on a LKB 1 ultratome. Sections were lifted off the glass knife with a camel hair brush or fine forceps and transferred to a small drop of water on a 3 x 1 inch glass slide. The sections were flattened by passing the slide through a bunsen flame and stained with alkaline toluidine blue.

4. Preparation of mosquitoes for autoradiography

(a) Injection of isotopes

After feeding on the blood of a domestic cat infected with microfilariae of Brugia patei, females of Aedes togoi and Anopheles labranchiae atroparvus were anaesthetised for 1-2 minutes in the cold drawer of a refrigerator and then each mosquito was given a single injection of approximately 0.23 µl (±0.02) of mosquito saline (Hayes, 1953) containing one of the isotopes listed below. Before injection each isotope was diluted with mosquito saline to equal a final concentration of 0.1 mCi/ml. The injection was accomplished by the use of a finely extended glass capillary pipette and the site of injection was the post spiracular membrane of the mesothorax. The mosquitoes recovered immediately after injection and were held for various times before fixation in FA.

To study the incorporation of labelled nucleosides in the parasite and its mosquito host, thymidine-6-[H₃] (specific activity 26 Ci/mmol) was injected into parasitised females of Aedes togoi at either 3, 6, 9, 12, 18, 21 or 24 hours post blood meal, or at 5, 6 or 8 days pbm. Further individuals together with infected Anopheles labranchiae atroparvus were injected at 5 days pbm. Injected mosquitoes were held for 30 minutes before fixation in FA, except for several infected females of Aedes togoi who were held for 60 minutes before fixation.

Uridine-5-[H₃] (specific activity, 24.0 Ci/mmol) was injected into parasitised females of Aedes togoi at either 26.5 or 45 hours pbm or at 5, 6 or 8 days pbm. Parasitised females of Anopheles labranchiae atroparvus plus additional females of Aedes togoi were injected at 45 hours pbm. All the uridine-[H₃] injected mosquitoes were held for 30 minutes before fixation in FA.

To study the incorporation of labelled carbohydrate into both parasite and host, parasitised females of Aedes togoi and Anopheles labranchiae atroparvus were given one of the following hexose sugars :-

D-glucose-6[H₃] (specific activity, 2.2 Ci/mmol) at 93 hours.
 D-glucosamine-6-[H₃] HCl (specific activity, 12.6 Ci/mmol) at 95 hours.
 D-(1-3H) galactose (specific activity, 13.0 Ci/mmol) at 95 hours.

For studies mainly on the formation of the host reaction of Anopheles labranchiae atroparvus arising around Brugia patei, infected mosquitoes were injected at either 24, 48 or 96 hours pbm with either glucose-[H₃] or galactose-[H₃] with specific activities as given above. After injection mosquitoes were held for 30 minutes before fixation in FA.

To study the incorporation of labelled amino acids into both parasite and host, parasitised females of Aedes togoi and Anopheles labranchiae atroparvus were each given an injection of one of the following amino acids at the following times pbm :-

L-histidine-2,5-[H₃] (specific activity, 58 Ci/mmol), given at 96 hours.
 L-proline-H₃(G) with specific activity of 0.54 Ci/mmol, given at 120 hours.
 L-arginine-5-[H₃], CH₁ (specific activity, 8.9 Ci/mmol), given at 97 hours.
 L-lysine-4, 5-[H₃](n) monohydrochloride (specific activity, 8.7 Ci/mmol) given at 92 hours.
 L-methionine (methyl-[H₃]) with specific activity of 9.0 Ci/mmol, given at 95 hours.
 L-cystine-3-H (specific activity, 2.4 Ci/mmol) given at 99 hours.
 L-isoleucine-4, 5-[H₃](n) with (specific activity of 30 Ci/mmol) given at 91 hours.
 L-leucine-4, 5-[H₃] (specific activity, 39 Ci/mmol) given at 93 hours.

L-valine-2, 3-[H₃](n) with specific activity of 39 Ci/mmol, given at 90.5 hours.

L-3-phenylalanine (ring-4-[H₃]) with specific activity of 10.5 Ci/mmol, given at 92 hours.

L-tyrosine-3, 5-[H₃] (specific activity Ci/mmol), given at 117 hours.

L-tryptophan-H₃(G) with specific activity of 5.0 Ci/mmol, given at 91.5 hours.

Dl-5-hydroxytryptophan-H₃(G) with specific activity of 7.1 Ci/mmol, given at 98 hours.

L-3 (3,4,-dihydroxyphenyl) alanine, ring 2,5,6-[H₃] with specific activity of 1.0 Ci/mmol, given at 92 hours.

The majority of the mosquitoes were pulsed for 30 minutes before fixation in FA, but some individuals of Aedes togoi that had been injected with either radioactive leucine, histidine, arginine, proline or tyrosine were held for 120 minutes before fixation in FA. A batch of infected females of Anopheles labranchiae atroparvus that had been injected with tyrosine-[H₃] were held for either 120, 240 or 350 minutes before fixation. A batch of infected Aedes togoi were injected with leucine-[H₃] having a higher specific activity (51.0 Ci/mmol) than used previously. In this latter experiment the mosquitoes were held for either 2, 5, 10, 20 or 30 minutes before fixation in FA. Some individuals of Anopheles labranchiae atroparvus were also injected with the hotter leucine-[H₃] at 96 hours pbm.

To record the incorporation of labelled amino acid into third stage larvae of Brugia patei, and into abnormal larvae of a corresponding age, females of Aedes togoi and Anopheles labranchiae atroparvus were injected with leucine-[H₃] (specific activity 51.0 Ci/mmol) at 11 days pbm. As before, mosquitoes were held for 30 minutes before fixation in FA.

For studies mainly on the formation of the host reaction of Anopheles labranchiae atroparvus developing around Brugia patei infected individuals of the latter mosquito were injected at either 24, 48, 72 or 96 hours pbm with the following :-

L-3 phenylalanine (ring-4H₃) with specific activity of 11.0 Ci/mmol, L-tyrosine 3,5-H₃ (specific activity, 49 Ci/mmol), L-3 (3,4-

dihydroxyphenyl) alanine (specific activity, 26.0 Ci/mmol), L-arginine 5-H₃ HCl (specific activity, 22.0 Ci/mmol). Radioactive phenylalanine and DOPA were also injected into uninfected individuals of Aedes togoi and Anopheles labranchiae atroparvus at 93 hours pbm, together with further infected individuals of the two species. All the above animals were pulsed for 30 minutes before fixation in FA.

(b) Staining and coating of autoradiographs

Following fixation, the mosquitoes were processed and embedded in paraplast as described previously. Paraplast sections were rehydrated then washed in further changes of distilled water for at least an hour before coating with emulsion. The majority of the autoradiographs were stained before coating with Bargman's modification of Gomori's chrome haematoxylin (Pearse, 1963) except that no mordant or oxidation step was employed. Staining was effective when used for 20-30 minutes followed by rinsing in 0.25% HCl in 70% ethanol (20-30 dips) and a thorough washing in running tap water. The slides were then returned to distilled water to await coating with the emulsion. Some autoradiographs were stained after coating and development with dilute Erlich's or modified Harris' haematoxylin (1:1 with distilled water) for 10-20 minutes (see Gahan, 1972).

Unstained or prestained slides were coated with Kodak AR10 stripping film (Gahan, 1972) after first floating out the film on sugar bromide water (Stephens, 1974) and within 20-30 minutes of coating were stored in light proof boxes, each containing a layer of silica gel. As an additional safeguard against fogging each box was either covered with black paper or sealed with black adhesive tape.

The boxes were then stored in a refrigerator set at 4°C throughout the period of exposure which was a standard 6 weeks \pm 2 days. However, some slides were exposed for only 4 weeks, others for 9 weeks. Development of the exposed slides was carried out at a temperature of 17-18°C for 6 minutes using D19b developer. The slides were washed in distilled water for 30 seconds and fixed in Johnsons' Fix-Sol (diluted 1 : 9 with distilled water) for 10 minutes. The slides were given an initial agitation of 10 seconds during each stage of development.

After development the slides were washed in running tap water for 10-15 minutes and allowed to dry.

Attempts to dehydrate the slides through graded ethanols followed by mounting in Euparal or clearing in xylene and mounting in balsam were consistently unsuccessful as air was usually retained in the specimens. This problem was overcome by mounting the slides from water into a water soluble mountant (Gurrs) diluted 1 : 1 with distilled water. In some slides, drained and left to dry, air was not retained in the specimen. In such cases grain counts taken over tissues were compared with grain counts collected from the same tissues after the slide had been mounted in the water soluble mountant. No significant difference was noted between the two sets of counts. Why some slides retain air in the specimen following dehydration through graded ethanols remains a mystery.

(c) Analysis of autoradiographs

The method used here to record the relative level of radioactivity between different sources was to employ a calibrated squared eye piece to obtain an estimate of the mean number of grains per unit area over a source. As the number of silver grains over a source include a contribution made by extraneous background radiation (see Rogers, 1973) the significance of the difference between the mean number of grains over a tissue and that over a much larger area of background was estimated, when necessary by a paired t test.

Grains over a source, whether tissue or background were accumulated as follows :-

A Zeiss photo-microscope was used at an optovar setting of 1.6, using a X8 eyepiece and X100 Oil. At this magnification the standard counting square used in the eye piece had an area of $64 \mu\text{m}^2$. For some tissues this represented too large a square, eg, cuticle of the mosquito. In such cases counting squares with an area of $16 \mu\text{m}^2$ were employed.

With stripping film, the silver grains in the exposed autoradiograph

lie in a different plane to the tissue, as the emulsion layer is relatively thick (Rogers, 1973). Thus selection of squares for counting was achieved by first focussing on the tissue, and choosing a square at random over it, the grains within the selected square were then brought into focus and counted. Five squares were used as a minimum sample size. In many tissues multiples of five squares were collected, but it was soon established that a mean estimated from a five square sample was adequate to determine whether a tissue or worm was labelled with respect to the background mean grain density.

The latter was estimated by counting unit areas over emulsion adjacent to the sample, and throughout further tissue spaces covered by emulsion until a mean derived from 100 unit areas of background was obtained.

5. Staining methods

(a) Routine

For observing features of general histological interest Bargman's modification of Gomori's chrome haematoxylin phloxine was used, with and also without permanganate oxidation (Pearse, 1968). Masson's trichrome and Mallory's triple stain were used as given in Culling (1963). Routine alum haematoxylin eosin techniques were used occasionally.

(b) Histochemical

Nucleic acids. Methyl green pyronin was used as modified by Kurnick (1955) in order to stain DNA and RNA. Nuclear chromatin stains green. The nucleoli and cytoplasmic RNA stain bright red.

Carbohydrates and mucosubstances. 1 : 2 glycol groups present in glycogen and other G mucosubstances (Pearse, 1968) were demonstrated by the Periodic acid-Schiff (PAS) technique, using Lillie's "cold Schiff" reagent and sodium metabisulphite rinses (Lillie, 1965). Lhotka's sodium bismuthate Schiff technique was used occasionally as an alternative to the PAS method (Lhotka, 1952) and demonstrates hydroxy-carboxylic acids in addition to 1 : 2 glycols. With these two techniques Schiff reactive sites stain magenta.

Glycogen was also demonstrated by Best's carmine (Pearse, 1968) and by toluidine blue staining of its sodium bisulphite addition derivatives (Malonin, 1970). Glycogen-deposits are stained bright red by Best's carmine and exhibit ethanol resistant metachromasia with the Malonin technique.

Acid mucosubstances were localised by using a number of polyanion detecting methods.

Alcian blue 8GX was used as a 1% solution in 3% acetic acid according to the directions of Mowry (1960). This technique designated as AB(2.5) stains carboxylated and sulphated mucosubstances bright blue. Sulphated mucosubstances were located by using Alcian blue 8GX at pH 1.0 ie AB (1.0) as directed by Lev and Spicer (1964). Alcian blue 8GX was also used at pH 5.7 but with varying concentrations of MgCl_2 added to the dye bath. This technique was used as directed by Scott and Doriing (1965). At concentrations of less than 0.3M. MgCl_2 staining due to $-\text{COO}^-$ and PO_4^- ceases but at levels greater than 0.3M. MgCl_2 only material containing polysulphates continue to stain blue.

The Low Iron Diamine (LID) and High Iron Diamine (HID) methods of Spicer (1965) were used according to revised instructions given in Culling (1974). With LID, sulphated and uronic acid containing mucosubstances stain grey to purple black whereas neutral mucosubstances stain purple-grey. The HID method stains sulphated mucosubstances black to purple black.

Proteins. Proteins rich in tyrosine were located using the Millon reaction as modified by Bensley and Gersh (Pearse, 1968) and the diazotisation coupling method of Glenner and Lillie (1959). Proteins rich in tyrosine stain rose red with Millons reagent and purplish red-pink with the coupling method.

Proteins rich in tryptophan were stained blue by the DMAB-nitrite method of Adams (1957). Basic proteins were stained a deep blue by the Mercuric bromphenol blue method (Barka and Anderson, 1963). Proteins rich in side chain carboxyls were localised by the mixed anhydride method of Barnett and Seligman (1958). Protein bound side chain carboxyls stain

a deep reddish purple colour.

Proteins rich in cystine were stained blue by per-acid Alcian blue procedures (see Lillie, 1965; Pearse, 1968) and those containing SH-groups were stained orange by the Mercury orange method of Bennet and Watts (Barka and Anderson, 1963).

Lipids. The Oil red O method of Lillie and Ashburn (Lillie, 1965) was used to locate neutral fat. Droplets of fat stain bright red.

To detect phospholipids, except for sphingomyelin, the copper phthalocyanine method was used (Pearse, 1968). Phospholipids stain blue.

Enzymes. Acid phosphatase activity was located by using the Naphthol AS and HPR technique (Pearse, 1968). The final reaction product is bright red.

Non-specific esterase activity was located as a deep blue reaction product when using the Naphthol AS-D acetate method (Pearse, 1968).

Acetyl-B-glucosaminidase activity was located by the Naphthol AS-BI-HPR method of Hayashi (1965). The final reaction product is a bright red deposit.

Pigments. A limited number of tests were used to identify the pigment component of the host reaction substance of Anopheles labranchiae atroparvus. Perl's prussian blue method (Lillie, 1965) was used to detect the presence of any iron in the pigment. The reaction product is bright blue. Masson's fontana method (Lillie, 1965) was used to identify a melanin component of the pigment. The reaction product is black. Attempts were made to dissolve the pigment with concentrated sulphuric, hydrochloric and peracetic acids.

Enzyme digestion and chemical blockades. Bovine pancreatic ribonuclease (4 x crystallised) was used to remove RNA from sections prior to methyl green pyronin staining. Slides were incubated in the enzyme solution (1 mg/ml distilled water) for 2 hours. Pyronin staining prevented by prior incubation of the sections in ribonuclease was held to be due to RNA.

α and β mixed amylase was used to remove glycogen from sections prior to PAS, Best's carmine or sodium bisulphite toluidine blue staining. Sections were incubated in a 1% solution of the enzyme in distilled water for 2 hours at room temperature (Barka and Anderson, 1963). Alternatively some slides were incubated in human saliva for 2 hours at 37°C in order to remove glycogen.

Aldehyde groups either native or engendered by periodate oxidation, were blocked by treatment with aniline in acetic acid (Lillie and Glenner, 1957). Sodium borohydride (Lillie and Pizzolato, 1972) or phenylhydrazine hydrochloride (Spicer, 1961) were occasionally used as alternative blocking agents.

1:2 glycol groups were blocked by overnight acetylation with acetic anhydride in pyridine (McManus and Mowry, 1960).

-SH groups were blocked by 0.1M n-ethyl maleimide in phosphate buffer (pH 7.4) for 4 hours at room temperature (Barka and Anderson, 1963).

-SS- groups in disulphide rich protein were blocked by prior reduction to -SH with sodium thioglycolate (Lillie, 1965). The -SH groups were then blocked by n-ethyl maleimide (Barka and Anderson, 1963).

6. Preparation of mosquito blood films

Attempts were made to remove haemolymph from uninfected females of Aedes togoi and Anopheles labranchiae atroparvus and also from these mosquitoes when infected with Brugia patei. Each mosquito was punctured through the side of the mesothorax with a fine glass pipette and a small volume of fluid withdrawn. By measuring the internal diameter of a batch of pipettes with a micrometer eye piece and measuring the maximum distance the fluid travelled up the pipettes, it was found that the volumes withdrawn did not exceed 1 μ l. The fluid had a tendency to clot in the pipette although it was usually possible to expell it onto unsubbed 3 x 1 glass slides. The smears were then fixed in 10% formalin or FA and stained for 2 hours with dilute giemsa (1:10) at a pH of 6.8. Upon inspection under the microscope the fluid was found to consist of haemolymph and tissue debris.

7. Melanisation/sclerotisation inhibitors

The compound (DL)-3-(3,4-dihydroxyphenyl)-2-hydrazino-2-methylpropionic acid, also known as α MDH or DOPA-carb, is a known DOPA decarboxylase inhibitor (Bodnaryk, 1970; Schlaeger and Fuchs, 1974a). Female Anopheles labranchiae atroparvus infected with Brugia patei were given a single injection at 24 hours pbm of α MDH dissolved in mosquito saline (Hayes, 1953). Two concentrations of α MDH were used, either 0.5 or 1.0 $\mu\text{g}/\mu\text{l}$. The site of each injection was the mesothorax and the volume administered was sufficient to extend the abdominal intersegmental membranes. Controls were injected with saline. After injection the mosquitoes recovered well and were maintained at 26°C with access to sugar pads. α MDH was also given orally to some uninjected individuals by being added to the sugar pad. In each experiment mosquitoes were killed at selected intervals after dosing and dissected to determine whether the degree of melanisation of the worms had decreased relative to the controls.

The compound phenylthiourea (PTU) has been successfully used to decrease the incidence of melanisation around parasites in insects (Salt, 1956; Brewer and Vinson, 1971; Nappi, 1973). PTU was given orally to female Anopheles labranchiae atroparvus infected with Brugia patei. The compound was added to the sugar pad to make a final concentration of either 1 or 10 μg PTU per 100 mls sugar solution. Mosquitoes were dissected at intervals after the infected feed to determine whether PTU had an effect on the incidence of melanised larvae.

8. Injection of DOPA and B-alanine

DOPA is a known substrate of melanin (Thomsen, 1962) and B-alanine is believed to reduce the polymerisation of melanin sub units (see Hodgetts and Konopka, 1973).

Anopheles labranchiae atroparvus infected with Brugia patei were given a single injection at 24 hours pbm of either DOPA or B-alanine dissolved in mosquito saline (Hayes, 1953). The concentration of the compound in the saline was 0.8 $\mu\text{g}/\mu\text{l}$. The site of each injection was the mesothorax and the volume given was sufficient to extend the abdominal intersegmental

membranes. The mosquitoes recovered well after the injections and were maintained at 26°C with access to sugar pads. The mosquitoes were dissected at intervals after the injections to determine whether the degree of melanisation of the worms had increased relative to saline injected controls.

9. Photography

Black and white photographs (Ilford Pan F) were taken with a Zeiss photomicroscope I. Exposed films were developed in Perceptol diluted 1:1 at 20°C for 19 minutes and fixed in Johnson's Fixol (1:3) for 10 minutes. Colour photographs (Agfachrome 50L) were taken with a Leitz Dialux microscope fitted with an Orthomat camera. Processing was done commercially.

PART 3THE DIFFERENTIAL INCORPORATION OF RADIOACTIVE NUCLEOSIDES, CARBOHYDRATES
AND AMINO ACIDS BY BRUGIA PATEI UNDERGOING NORMAL DEVELOPMENT IN AEDES
TOGOI AND ABNORMAL DEVELOPMENT IN ANOPHELES LABRANCHIAE ATROPARVUSINTRODUCTION

In the mosquito Aedes togoi, the filarial nematode Brugia patei moults to the second stage larva between 4-5 days following the entry of the microfilaria into the mosquito, and the moult to the third stage, occurring between 7-10 days is followed by migration of the infective larva from the thoracic flight muscle into the haemocoel of the mosquito (Laurence and Pester, 1967; Laurence and Simpson, 1971). These latter workers showed that development of the larva during the first 4-5 days results in the differentiation of the pharynx from its modified form present in the microfilaria, and of the development of the intestine from the cells around the inner body. After the moult to the second stage the larva of Brugia is capable of active feeding, being able to ingest solid fragments of host origin (Beckett and Boothroyd, 1970; Laurence and Simpson, 1971). Ho and Kan (1972) working with Breintzia sergenti reported that the infective larva of this filarial nematode can ingest tissue fragments derived from its mosquito host. In contrast to these results little is known about the nutritional requirements of the first stage filarial larva shortly after its entry into the intermediate host. The only information currently available on the metabolic activity of the microfilarial form of the first stage larva is the work of Langer and Jirampermpoon (1970), and Jaffe and Doremus (1970), both groups working with the blood borne microfilariae of Dirofilaria immitis.

In the present study, this particular section deals with the uptake of nucleosides, carbohydrates and amino acids in normal larvae, and in abnormal larvae present within the refractory mosquito.

In the microfilaria, the evidence available to date suggests that the G cell is the first cell to undergo mitotic division, and in Brugia it divides within 24 hours of entry of the parasite into the vector

(Feng, 1936; Schacher, 1962a; Laurence and Simpson, 1971). Following the division of the G cells the cells immediately anterior and posterior to it, and which had been separated by its presence grow towards each other, eventually forming the gut (Laurence and Simpson, 1971). The G cell is thus a cell of strategic importance as its failure to divide would effectively inhibit the succeeding programmed sequence of cell divisions that result in the differentiation of the alimentary canal.

Aedes togoi, infected with Brugia patei were injected with thymidine-[H₃] at selected time intervals within the period 3-24 hours following entry of the parasite into the mosquito. The object of this was to provide the nucleoside pool of the mosquito with available isotope that would become incorporated into the DNA of the parasite, when the latter entered the S phase of DNA synthesis. By labelling the G cell in this manner, the intention was then to determine the fate of its progeny, as there remains disagreement regarding the fate of this cell (see Laurence and Simpson, 1971).

RNA synthesis by Brugia patei was investigated by injecting Aedes togoi with uridine-[H₃] at various time intervals following entry of the parasite into the host. To determine whether abnormal larvae of Brugia patei could also incorporate these nucleosides, infected females of Anopheles labranchiae atroparvus were also injected with either of the two isotopes.

The third stage larva of Brugia pahangi was shown by Collins (1971) to contain droplets of lipid and rosettes of glycogen in the body musculature. In the adults of Brugia pahangi and Brugia malayi the cells of the lateral chords and somatic musculature contain storage depots of glycogen (Vincent, Portaro and Ash, 1975; Vincent, Ash and Frommes, 1975). In the microfilaria, ultrastructural studies carried out on a number of species (Kozek, 1971; McLaren, 1972; Laurence and Simpson, 1974; Tongu, 1974) indicated that only small amounts of glycogen are present. At the light microscope level, Simpson and Laurence (1972) did not record the presence of sizable deposits of this storage polymer. Jaffe and Doremus (1970) reported that the microfilaria of Dirofilaria immitis used C₁₄ labelled glucose as a source of energy. Sometime then, during the period of development in the intermediate host, glycogen

synthesis in the filarial larva must increase to the point when reserves of this polymer start to accumulate.

To determine whether Brugia patei could accumulate glycogen as early as the first larval stage, and whether there was any recognisable incorporation of sugar into the carbohydrate components of the first stage larva, [H_3]-labelled glucose, galactose and glucosamine were injected into parasitised females of Aedes togoi and Anopheles labranchiae atroparvus at times of up to 4 days pbm. In parallel with this, histochemical techniques used to locate glycogen and other carbohydrates were carried out on all three larval stages, including larvae in the refractory host.

To study amino acid uptake by Brugia patei, 14 different amino acids were injected, one per animal, into both mosquito hosts during the period 4-5 days pbm. These amino acid experiments were expected to indicate whether the pattern of amino acid incorporation by the parasite was radically different when developing abnormally in the refractory host.

RESULTS

1. Nucleoside incorporation in *Brugia patei*

No significant incorporation of thymidine-[H₃] was observed in first and second stage larvae undergoing normal development. Among the many infective stage larvae that were examined only a single individual worm was observed to have specific marking over a nucleus that was present in one of the cells of the body wall. Abnormal and melanised larvae were also unlabelled with respect to background radioactivity. In total, autoradiographs from 56 females of *Aedes togoi* and 1 female of *Anopheles labranchiae atroparvus* were examined in the thymidine-[H₃] experiment. Many worms were present in these animals. The single worm showing thymidine-[H₃] incorporation into one nucleus represented less than 1% of the total number of worms examined. At this level of labelling, the marking seen in one worm was considered to be insignificant. In contrast both normal and abnormally developing larvae showed a significant incorporation of the nucleoside uridine-[H₃]. Incorporation was quite marked in the first stage larva (45 hours-5 days) compared to the second and third larval stages, (Table 1). In the first stage larva, the mean grain density obtained for individual worms was particularly variable at 26.5 hours pbm. Out of a total of 10 worms sampled, 4 were not significantly labelled relative to background radioactivity. The maximum grain count recorded for an individual larva at this time period was 20.80 ± 2.91 . The minimum mean grain density recorded among the remaining worms in this sample was 8.0 ± 1.46 . At 45 hours and 5 days following the entry of the parasite into the host, variation in the level of marking between individual worms was comparatively less. At 45 hours, only 1 worm from a total of 10 sampled was found to be unlabelled with respect to background. The individual means of the labelled worms lay between 10.0 ± 1.41 to 21.80 ± 2.42 . At 5 days all of the 8 worms sampled were labelled, the individual means lying between 7.60 ± 1.08 to 23.60 ± 2.79 .

In the larvae sampled from the refractory host, the individual means obtained for 2 larvae from a female injected at 45 hours, were 25.20 ± 2.50 and 41.20 ± 7.39 .

Table 1 Incorporation of uridine-[H₃] into Brugia pateri

Time injected (pbm)	No mosquitoes used	No worms sampled	Mean grain density \bar{x} ± standard error of mean	
			Worm	Background
(a) Worms developing in <u>Aedes togoi</u>				
26.5 hours	1	10	8.78±6.00	2.03±0.10
45 hours	1	10	15.64±1.00	1.79±0.11
45 hours (replicate)	1	4	17.25±1.80	4.50±0.10
5 days	1	8	15.15±1.00	1.90±0.10
6 days	1	9	5.00±4.00	1.96±0.15
8 days	1	10*	4.80±0.26	1.97±0.15
(b) Worms developing in <u>Anopheles labranchiae atroparvus</u>				
45 hours	1	2	33.20±4.99	4.70±0.20

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x})
per unit area of worm = $\frac{(\text{total number grains counted})}{\text{number worms sampled} \times 5 \text{ unit areas per worm}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{(\text{total number grains counted})}{100 \text{ unit areas}}$

* In the 8 day mosquito the worms had migrated into the haemocoel, which in sections, made it impossible to distinguish individual worms. 10 x 5 unit areas were randomly sampled from the mass of worm sections present in the haemocoel.

The 45 hour replicate Aedes togoi was processed with the 45 hour Anopheles labranchiae atroparvus.

1 unit area = 64 μm^2 .

In second stage and infective larvae examined at 6 and 8 days respectively, lower incorporation of uridine-[H₃] was found (Table 1). At 6 days (Table 1) 4 worms out of a total of 9 sampled were not significantly labelled relative to the background mean. The range of the individual means for the remainder lay between 5.6 ± 0.92 6.4 ± 1.43 . At 8 days, although it was not possible to distinguish individual worms in the sections, all the pieces of infective worm examined were labelled with respect to background.

Some attempt was made to assess the distribution of radioactivity within broadly recognisable components of individual worms. In early first stage larvae (26.5 hours, 45 hours pbm) uridine-[H₃] label was uniform throughout the tissues, but in the late first stage and in second stage larvae (ie between 4-6 days pbm) uridine-[H₃] label was observed to lie discreetly over the nucleoli of some cells, with many cells exhibiting little or no label. Ideally for a specified area the number of cells marked with nucleolar label could be scored along with those not showing label. The percentage of cells showing discreet uridine-[H₃] incorporation could be determined for the pharynx, intestine and body wall. This would, in the absence of thymidine marking, be a way of measuring the relative changes of cellular activity in these tissues. In the autoradiographs prepared from the uridine-[H₃] injected animals the quality of staining was poor. It proved too difficult to pursue the analysis quantitatively.

Some of the sections prepared from the uridine-[H₃] injected animals were treated with ribonuclease prior to coating with AR10 stripping film. Control sections were immersed in distilled water only. After routine exposure and development, the label over the tissues of the worms in the control sections was comparable to that observed in untreated sections. In the sections treated with ribonuclease, no significant labelling of the worms was observed with respect to background. It was concluded that uridine-[H₃] was being incorporated into RNA, and that the incorporation of uridine-[H₃] was at a maximum in the 1st stage larva.

2. Carbohydrate incorporation in *Brugia patei*

No significant incorporation with respect to background radioactivity was observed in *Brugia patei* in *Aedes togoi* and *Anopheles labranchiae atroparvus* when exposed to glucosamine-[H₃], glucose-[H₃] and galactose-[H₃] at 4 days pbm (Table 2).

In a second experiment *Anopheles labranchiae atroparvus* parasitised with *Brugia patei* was injected with glucose-[H₃] or galactose-[H₃] at 24, 48, 72 and 96 hours pbm. No significant label with respect to background radiation was found over worms in the galactose-[H₃] injected mosquitoes but the larvae in the glucose-[H₃] injected mosquitoes appeared to be slightly labelled (Table 2).

3. Amino acid incorporation in *Brugia patei*

At the onset of this investigation into amino acid uptake by *Brugia patei*, the decision was taken to broadly study the relative incorporation of a range of [H₃]-labelled amino acids, rather than an in-depth study of just one or two. As 14 amino acids were scheduled for use, initial studies were directed towards establishing the length of the pulse which would be adequate to record whether an amino acid was going to be taken up by the parasite. With [H₃]-leucine, females of *Aedes togoi* were killed at 2, 5, 10, 20 and 30 minutes after a single injection of the isotope. A comparison of the mean grain density over larvae sampled from these mosquitoes confirmed that a pulse of 20-30 minutes duration would be very suitable (Table 3). No worms were observed in animals killed 2 minutes after injection but label was found in larvae in the muscle after 5 minutes.

Tritium labelled amino acids, histidine, proline and tyrosine were injected into parasitised females of *Aedes togoi* and the mosquitoes were sacrificed 30 minutes or 120 minutes later. One amino acid was injected per animal. Tyrosine-[H₃] was injected into parasitised females of *Anopheles labranchiae atroparvus* and the mosquitoes were killed at 30 minutes, 2 hours, 4 hours and 6 hours later. Grain counts were made on larvae sampled from each of the mosquitoes injected. With these amino acids, the mean grain density obtained over one or more

Table 2 Incorporation of $[H_3]$ -carbohydrate in *Brugia patei* undergoing (a) normal development in *Aedes togoi* (b) abnormal development in *Anopheles labranchiae atroparvus* during the period 1-4 days pbm

Isotope	Time of injection (hours pbm)	No mosquitoes used	No worms sampled	Mean grain density \bar{x} \pm standard error of mean	
				Worm	Background
(a) Worms developing in <u>Aedes togoi</u>					
Glucosamine	95	1	2	0.90 \pm 0.22	0.86 \pm 0.09
Galactose	92.5	1	2	1.30 \pm 0.40	1.03 \pm 0.10
(b) Worms developing in <u>Anopheles labranchiae atroparvus</u>					
Glucose	24	3	8	7.30 \pm 0.96	1.96 \pm 0.18
	48	2	5	5.04 \pm 0.65	2.77 \pm 0.17
	72	2	4	8.15 \pm 1.16	1.60 \pm 0.14
	96	3	10	4.38 \pm 0.34	3.09 \pm 1.19
Galactose	24	1	1	1.40 \pm 1.04	3.39 \pm 0.22
	48	1	3	2.60 \pm 0.87	6.18 \pm 0.36
	72	1	2	4.30 \pm 0.81	5.53 \pm 0.25
	96	1	2	2.00 \pm 0.71	1.80 \pm 0.10
Glucosamine	95	1	2	2.90 \pm 0.35	1.74 \pm 0.16
Galactose	92.5	1	1	2.60 \pm 0.60	1.03 \pm 0.09

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x}) per unit area of worm = $\frac{(\text{total number grains counted})}{\text{number worms sampled} \times 5 \text{ unit areas per worm}}$

Mean grain density (\bar{x}) per unit area of background = $\frac{(\text{total number grains counted})}{\text{number mosquitoes used} \times 100 \text{ unit areas}}$

1 unit area = 64 μm^2 .

worms for each of the pulse periods used is shown in Table 3. There appeared to be little advantage to be gained by extending the pulse beyond 30 minutes. Thereafter the decision was made to select 30 minutes pulse as a standard treatment in future experiments.

(a) Uptake of labelled amino acid between days 4-5

The relative degree of incorporation of radioactive amino acids by normal and abnormal worms between days 4-5 pbm is set out in Table 4. The observed degree of incorporation relative to background radiation varied with the amino acid used (Plate 1) ranging in normal worms from relatively high levels of uptake with iso-leucine-[H₃], phenylalanine-[H₃], leucine-[H₃] and tryptophan-[H₃] to significant incorporation of histidine-[H₃] ($p < 0.05$) and proline ($p < 0.05$). Normal worms did not incorporate significant amounts of OH-tryptophan-[H₃] ($p < 0.01$), and DOPA-[H₃] ($p < 0.05$).

This pattern of labelled amino acid incorporation was only partly matched by worms undergoing varying degrees of abnormal development in Anopheles labranchiae atroparvus. In this mosquito Brugia patei incorporated comparatively little tyrosine-[H₃], cystine-[H₃] and tryptophan-[H₃] in relation to that observed for normal worms of the same age. The mean grain density recorded for abnormal worms taking up tyrosine was particularly low, but still significant in relation to background label ($p < 0.05$). A further difference noted in respect of amino acid uptake by normal and abnormal larvae was that the latter incorporated relatively high levels of valine-[H₃].

(b) Uptake of labelled arginine and phenolic amino acids in abnormal worms

In this next series of experiments, parasitised females of Anopheles labranchiae atroparvus each received a single injection of one of the following tritium labelled amino acids: phenylalanine, tyrosine, DOPA and arginine. Within each amino acid group, females were injected at 24, 48, 72 and 96 hours pbm. The mean grain densities recorded over worms sampled at each time interval are set out in Table 5. In Anopheles labranchiae atroparvus, Brugia patei does not usually exhibit abnormalities in its growth until after the G cell has divided,

Table 3 The effect of changing the duration of the radioactive pulse on [H₃]-amino acid incorporation in *Brugia patei*

Isotope	Time of injection (hours pbm)	Length of pulse (mins)	No worms sampled	Mean grain density \bar{x} ± standard error of mean	
				Worm	Background
(a) Worms developing in <i>Aedes togoi</i>					
Histidine	96	30	4	4.15±0.15	2.35±0.13
		120	10	9.86±0.64	2.61±0.12
Proline	120	30	2	3.60±0.72	0.83±0.09
		120	1	17.20±2.26	2.97±0.13
Tyrosine	117	30	3	19.20±1.02	2.83±0.74
		120		28.00±1.67	2.78±0.19
Leucine	96	5	3	7.73±3.12	<1
		10	6	30.06±5.01	<1
		20	1	47.0 ±3.54	<1
		30	1	>100	2.71±0.31
(b) Worms developing in <i>Anopheles labranchiae atroparvus</i>					
Tyrosine	117	30	3	6.30±1.24	2.23±0.10
		120	3	21.46±1.97	2.24±0.10
		240	3	6.26±1.12	2.00±0.15
		360	3	7.13±1.05	2.76±0.13

One mosquito of each species was used for each of the pulse periods shown.

Mean grain density (\bar{x})
per unit area of worm = $\frac{\text{(total number grains counted)}}{\text{number worms sampled} \times 5 \text{ unit areas per worm}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{\text{(total number grains counted)}}{100 \text{ unit areas}}$

1 unit area = 64 μm^2 .

(Laurence and Simpson, 1971; Oothuman, Simpson and Laurence, 1974). Yet, even at 48, 72 and 96 hours pbm when the worms were abnormal, they were still able to incorporate significant amounts of labelled phenylalanine, tyrosine and argine, but not DOPA-[H₃]. At 24 hours pbm DOPA-[H₃] incorporation into the larvae was significant relative to background radioactivity ($p < 0.05$).

(c) Uptake of leucine-[H₃] in third stage larvae

Leucine-[H₃] was injected into females of parasitised Aedes togoi and Anopheles labranchiae atroparvus at 11 days pbm. In Aedes togoi the mean grain density recorded over 5 worms was 22.80 ± 2.20 . In Anopheles labranchiae atroparvus, the mean grain density recorded over 2 abnormal worms which had not succeeded in developing beyond the first larval stage was 63.0 ± 3.56 . The mean grain density due to background radioactivity was less than 1 in both mosquitoes.

4. Histochemistry of larval Brugia patei

(a) Nucleic acids

Staining was carried out on carnoy fixed sections of Brugia patei in Aedes togoi at 1-8 days pbm and in Anopheles labranchiae atroparvus at 48 hours pbm. In the microfilaria, pyronin staining was confined to the cell nucleoli, the G cell, R cells, excretory cell and anal vesicle, whereas at 48 hours strong pyronin staining was present throughout the tissues of both normal and abnormal larvae. Strong pyronin staining was also present in larvae at 3-6 days pbm, but injected larvae at 7-8 days had little affinity for the dye. Pyronin staining of the larvae was prevented by a prior incubation of the sections in ribonuclease, indicating that the red staining was due to RNA.

(b) Glycogen

Staining was carried out on carnoy, BD and FA fixed sections of Brugia patei in Aedes togoi at 1-8 days pbm and in Anopheles labranchiae

Table 4 Incorporation of [H₃]-amino acid into late first stage to early second stage larvae of *Brugia patei*

Isotope	Time of injection (hours pbm)	No worms sampled	Mean grain density \bar{x} ± standard error of mean	
			Worm	Background
(a) Worms developing in <u>Aedes togoi</u>				
Histidine	96	4	4.15± 0.50	2.35±0.13
Proline	120	2	3.60± 0.72	0.83±0.09
Arginine	97	1	10.60± 1.93	3.28±0.18
Lysine	92	1	38.20± 2.79	<1
Methionine	95	1	14.00± 1.81	1.99±0.14
Cystine	99	3	33.50± 4.0	5.21±0.23
Iso-leucine	91	1	121.00± 6.57	<1
Leucine	93	1	>100	2.71±0.31
Valine	90.5	1	24.80± 1.21	<1
Phenylalanine	92	3	57.13± 6.48	3.02±0.15
Tyrosine	117	3	19.20± 1.0	2.83±0.74
Tryptophan	91.5	2	83.0 ±10.15	<1
OH-tryptophan	98	3	4.80± 0.45	4.42±0.18
DOPA	93	4	2.31± 0.62	0.52±0.05
(b) Worms developing in <u>Anopheles labranchiae atroparvus</u>				
Histidine	96	3	5.30± 0.62	2.76±0.12
Proline	120	4	7.70± 0.47	2.02±0.13
Arginine	97	3	13.53± 1.77	2.56±0.13
Lysine	92	3	89.93± 4.28	<1
Methionine	95	2	5.90± 1.44	0.82±0.08
Cystine	99	3	17.26± 1.95	3.80±0.25
Iso-leucine	91	5	95.2 ±19.78	<1
Leucine	93	4	43.44± 2.71	2.27±0.13
Valine	90.5	10	63.00± 3.56	<1
Phenylalanine	92	2	40.86± 6.61	3.44±1.19
Tyrosine	117	2	6.30± 1.24	2.23±0.11
Tryptophan	91.5	10	13.20± 0.82	<1
OH-tryptophan	98	2	8.00± 0.99	4.41±0.15
DOPA	93	9	2.31± 0.62	1.16±0.47

One mosquito of each species was used for each amino acid.

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x})
per unit area of worm = $\frac{(\text{total number grains counted})}{\text{number worms sampled} \times 5 \text{ unit areas per worm}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{(\text{total number grains})}{100 \text{ unit areas}}$

Note It was impractical to sample an equal number of worms from each mosquito.

1 unit area = 64 μm^2 .

atroparvus at 24-96 hours pbm. With PAS, Best's carmine and sodium bisulphite toluidine blue techniques, staining that was prevented by a prior incubation in either human saliva or the mixed amylase solution was held to be glycogen. The latter was not present in normal larvae in Aedes togoi at 1-4 days pbm but was present in the body wall and gut of larvae at 5-8 days pbm. Abnormal larvae in Anopheles labranchiae atroparvus at 24-96 hours pbm also did not stain for glycogen. It should be emphasised that here, positive staining in the tissues of the larvae was held to be a strong, clear staining of particulate or flocculent material removable by amylase or saliva. The presence of trace amounts of colour in the larvae was ignored as this could be due to glycogen leaching from surrounding flight muscle.

(c) Proteins

Bromphenol blue was used to detect the presence of any protein storage material in carnoy, BD and FA fixed sections of Brugia patei in Aedes togoi and Anopheles labranchiae atroparvus at 1-11 days pbm. Other than the cell nuclei which continued to stain throughout the period 1-11 days pbm, only the small spheres of the inner korper were reactive to bromphenol blue. The inner korper had virtually disappeared by the second day pbm.

Table 5 Incorporation of phenylalanine-[H₃], tyrosine-[H₃], DOPA-[H₃] and arginine-[H₃] into *Brugia patei* when present in the flight muscles of *Anopheles labranchiae atroparvus*, during the period 1-4 days pbm

Isotope	Time of injection (hours pbm)	No mosquitoes used	No worms sampled	Mean grain density \bar{x} \pm standard error of mean	
				Worm	Background
Phenylalanine	24	1	2	10.10 \pm 3.05	<2
	48	1	2	76.30 \pm 9.84	3.33 \pm 0.29
	72	1	2	70.80 \pm 12.46	3.11 \pm 0.19
	96	1	2	72.27 \pm 8.31	<2
Tyrosine	24	2	7	17.23 \pm 1.24	1.72 \pm 0.27
	48	2	3	18.67 \pm 2.80	1.10 \pm 0.15
	72	2	7	54.94 \pm 3.14	2.83 \pm 0.87
	96	1	1	20.80 \pm 0.65	<1
DOPA	24	2	4	5.15 \pm 0.87	1.17 \pm 0.17
	48	2	3	2.80 \pm 0.52	1.09 \pm 0.16
	72	2	4	1.50 \pm 0.31	1.03 \pm 0.10
	96	2	5	3.0 \pm 0.51	<1
Arginine	24	2	7	25.10 \pm 2.11	<2
	48	2	4	12.05 \pm 1.57	<2
	72	2	6	22.20 \pm 4.88	<2

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x}) per unit area of worm = $\frac{(\text{total number grains counted})}{\text{number worms sampled} \times 5 \text{ unit areas per worm}}$

Mean grain density (\bar{x}) per unit area of background = $\frac{(\text{total number of grains counted})}{\text{number mosquitoes used} \times 100 \text{ unit areas}}$

Note It was impractical to sample an equal number of worms from each mosquito.

1 unit area = 64 μm^2 .

DISCUSSION

The inability of Brugia patei to sequester thymidine-[H₃] from the haemocoel of the susceptible mosquito Aedes togoi and the refractory mosquito Anopheles labranchiae atroparvus has been established. Omar and Gwadz (1974) reported a similar lack of incorporation of thymidine-[H₃] by Brugia pahangi parasitic in the susceptible (Liverpool Black Eye) strain of Aedes aegypti.

Although thymine is one of the four bases that enter into the construction of the DNA molecule, it is known that not all animal cells accept its deoxyriboside thymidine as a precursor from the extracellular environment (Feinendegen, 1967).

Jaffe and Doremus (1970) found that blood borne forms of the microfilaria of Dirofilaria immitis were, in vitro, unable to incorporate nucleic acid precursors into DNA. This latter work with the exception of biochemical studies on microfilarial enzymes represents our only source of biochemically derived information on the microfilaria and in this respect the importance of this work cannot be understated. However, it is considered here that the microfilaria represents a resting stage of the first stage larva. It undertakes no growth within the bloodstream of the vertebrate host and its uptake of raw materials may be expected to reflect its demands for energy (see Jaffe and Doremus, 1970). Its nutritional requirements may well be expected to differ upon entry into the intermediate host. It may be significant that other workers involved in studies on the uptake of nutrients by blood-borne parasitic protozoa have also reported a similar inability of these parasites to incorporate certain nucleic acid derivatives (Walsh and Sherman, 1968; Neame, Brownbill and Homewood, 1974).

The failure to utilise thymidine-[H₃] does not indicate that Brugia patei does not synthesise DNA, particularly as the times at which the host mosquito was injected with labelled thymidine corresponded to the times when the larva is mitotically active (Laurence and Simpson, 1971). It seems likely that the necessary precursors for DNA synthesis in Brugia patei are synthesised without the involvement of a thymidine pathway.

When thymidine enters a cell it is phosphorylated by the enzyme thymidine kinase prior to entering the pathway leading to DNA synthesis. It is possible that the larva of Brugia patei does not possess this enzyme. It is known that thymidine kinase can be synthesised de novo just prior to the commencement of DNA synthesis, or become activated in those situations where it is stored in an inactive form (Feinendegen, 1967). It could be argued that thymidine kinase is present in Brugia patei, but that it is, or becomes active for only a very short duration, and was inactive at the time of the radioactive pulse. In Aedes togoi the animals were sacrificed every 3 hours during the first 24 hours that followed the entry of the parasite into the mosquito. Each animal was pulsed for 30 minutes before killing. With such time intervals it is considered doubtful that the S phase of DNA synthesis would have been missed. Omar and Gwadz (1974) allowed their mosquitoes to feed ad libitum on 5% Karo syrup solution containing 50 $\mu\text{Ci/ml}$ of thymidine-methyl- $[\text{H}_3]$ (specific activity 6.7 Ci/mM) and still larval Brugia pahangi in the flight muscles remained unlabelled.

With the pyrimidine nucleoside uridine, the pattern of labelling was quite different. Here, Brugia patei incorporated uridine- $[\text{H}_3]$ during all three larval stages. The maximum intensity of label was recorded from Aedes togoi and Anopheles labranchiae atroparvus that had been injected at 45 hours pbm. As the size of the mosquito sample was so small, variation existing between individuals injected at the same time interval could not be measured. Clearly the significance of the differences observed between individuals injected at different time intervals during the period 1-8 days pbm requires verification. Meanwhile it is tentatively concluded that the low degree of label over the worms at 26.5 hours pbm represents a low uridine requirement for the larva at this time. The histochemical data supports this conclusion. Ribonuclease-labile pyronin staining of the larvae shows the greatest intensity at 48 hours pbm, and little at 24 hours. Also from 24 hours pbm the worms begin to show marked histological changes from the basic microfilarial pattern. It would be expected that this structural remodelling of the parasite would be accompanied by a rise in nucleic acid synthetic activity.

One fact has clearly emerged. The failure of Brugia patei to develop normally in Anopheles labranchiae atroparvus is not due to an inability

of the parasite to incorporate uridine-[H₃] into RNA. That the uridine-[H₃] label over the cells of the larva and those of the mosquito represented that incorporated into RNA, was indicated by the absence of label in those autoradiographs that had been treated with ribonuclease prior to coating with AR10 emulsion. It is concluded that during the period spent within the intermediate host, Brugia patei is dependent upon an endogenous source of pyrimidines for DNA synthesis, whereas an exogenously derived pyrimidine such as uridine becomes incorporated into RNA.

An interesting question to consider is whether Brugia patei is capable of the de novo synthesis of purines. Unfortunately nothing is known regarding the presence or absence of enzymes in this nematode, which are involved with nucleic acid synthesis. In other nematodes a little information is available concerning folate metabolism. Jackson and Platzer (1974) provided evidence suggesting that the entomophilic nematode Neoplectana glaseri metabolised folate, indicating that this nematode undergoes de novo purine and pyrimidine biosynthesis. Several enzymes involved in folate metabolism were found in the nematodes Aphelenchus avenae and Nippostrongylus brasiliensis (Platzer, 1974). Dihydrofolate reductase activity was detected in crude extracts of adult Dirofilaria immitis, Litomosoides carinii, Dipetalonema vitei and Onchocerca volvulus (Jaffe, McCormack and Meymarian, 1972).

In the case of filarial nematode larvae, the evidence to date suggests that they depend upon an endogenous source of purines. Omar and Gwadz (1974) observed little incorporation of adenine-[H₃] by either developing or mature larvae of Brugia pahangi parasitic in Aedes aegypti and Jaffe and Doremus (1970) found that the microfilaria of Dirofilaria immitis was unable to synthesise purine nucleotides from glycine and formate.

Enzymes involved in folate metabolism occur in mosquito flight muscle (Ventors, 1972), and it is interesting to speculate whether there is a relationship between folate synthesis in mosquito flight muscle and the purine requirements of filarial species which develop at this site. It would also be of interest to determine whether there are significant differences in the way the filarial larva meets its nucleic acid synthetic needs in comparison to that carried out by the adult.

Platzter (1974) has commented that blood-borne parasites may have achieved a reduction of biochemical mechanisms that have become superfluous as a result of the parasite adapting to an environment containing a readily available source of preformed purines.

There was no evidence to suggest that glucosamine-[H₃] or galactose-[H₃] were incorporated by Brugia patei whilst present within the flight muscles of the mosquito but the autoradiographs prepared from glucose-[H₃] injected mosquitoes were more difficult to interpret. As a result of poor fixation the leaching of glycogen out of the muscle fibres adjacent to the worm into the area occupied by the worm could explain the apparent relationship between glucose-[H₃] label of the parasite and of the flight muscle. Poorly labelled flight muscle had poorly labelled worms. The converse applied. Furthermore, in first stage larvae of Brugia patei, there appeared to be negligible amounts of PAS and Sodium-bisulphite-toluidine blue stainable material that was susceptible to amylase treatment. In contrast these techniques readily demonstrated glycogen in infective and even late second stage larvae. It seems unlikely that the first stage larva undertakes glycogen synthesis to the point of storage. It is quite likely that the first stage larva utilises glucose mainly as an energy source.

First stage larvae of Brugia patei undergoing normal development in Aedes togoi incorporated the tritium labelled amino acids, arginine, lysine, methionine, cystine, isoleucine, leucine, valine, phenylalanine, tyrosine and tryptophan. Uptake was exceptionally intense with radioactive iso-leucine, leucine, phenylalanine and tryptophan. Abnormal worms in Anopheles labranchiae atroparvus also incorporated these amino acids, except that in the animals under study, uptake by methionine-[H₃], tyrosine-[H₃] and tryptophan-[H₃] was low compared to normal worms of the same age, but alternatively, uptake by valine-[H₃] and lysine-[H₃] was higher. Normal and abnormal worms showed only a slight incorporation of histidine-[H₃] and proline-[H₃], and no significant incorporation of OH-tryptophan-[H₃].

In abnormal worms, significant but variable incorporation of amino acids occurred at 24, 48, 72 and 96 hours pbm. The mean grain density over worms that had been exposed to the isotope at 24 hours pbm was never intense

but at other time intervals, the mean grain density over some worms was high relative to that at 24 hours pbm. Clearly the host reaction does not impair the ability of abnormal larvae to take up radioactive amino acid from the haemolymph.

In normal worms using leucine-[H₃] as a marker, it was found that the third stage larva also incorporated leucine-[H₃] but in ten individuals sampled, the level of incorporation was lower than that recorded for the first stage larvae. In the refractory host, many worms were still alive at 11 days pbm. In these individuals the level of incorporation was still high. The reduced incorporation of radioactive amino acid by the infective larvae would be consistent with them having reached a stage where they have switched off further development pending transfer to the definitive host, whereas the abnormal larvae have not reached this stage and still continue to take up high levels of radioactivity.

Among different worms in the same host, some variation in incorporation of a particular amino acid would be expected, as development of filarial larvae in the vector is asynchronous (Laurence and Pester, 1961). Sampling error would also be a factor. A possibly significant feature to consider is whether the metabolic activity of the host mosquito exerts an influence on the availability of precursors to the parasite. Any individual variation between mosquitoes, in respect of their metabolic activity, may represent a contributing factor in the differences seen in amino acid uptake by worms in different mosquitoes. Although it is tempting to seek a precise meaning to the observed differences in the relative degree of amino acid uptake between worms developing normally and those showing restricted and abnormal development, the data is insufficient to permit this. However it is possible to note a trend which suggests that the 24 hours stage larva, whether destined to develop normally or not, has variable requirements for labelled uridine and amino acids compared with the sausage stage and later forms of the first stage larva, and second and third stage larvae.

During the period 24-96 hours pbm, incorporation of labelled metabolites is probably uniform, throughout the larva. In the later stages, incorporation is more selective.

The protein histochemical techniques failed to locate any heavily stained regions or cells which could be regarded as protein storage areas, other than the innen korper. It may be significant that by the time the latter has broken up and virtually disappeared by 48 hours pbm (Laurence and Simpson, 1971), the larva has been actively incorporating precursors from the host haemolymph. Conversely at 24 hours pbm the innen korper is still present as a series of small spheres which stain, albeit irregularly with histochemical methods for protein. In the microfilaria, Simpson and Laurence (1972) concluded that the innen korper contained a basic protein and acidic mucosubstance complex. Laurence and Simpson (1971; 1974) established that the innen korper represents the lumen of the intestine of the later developing, larval stages, and as such thought that this body served as a food store which becomes increasingly depleted during the early period of development of the first stage larva. The autoradiographic data presented here encourages such a line of thought.

The mechanisms which enable metabolites such as nucleosides and amino acids to cross through the cuticle and body wall of the larva remain unknown.

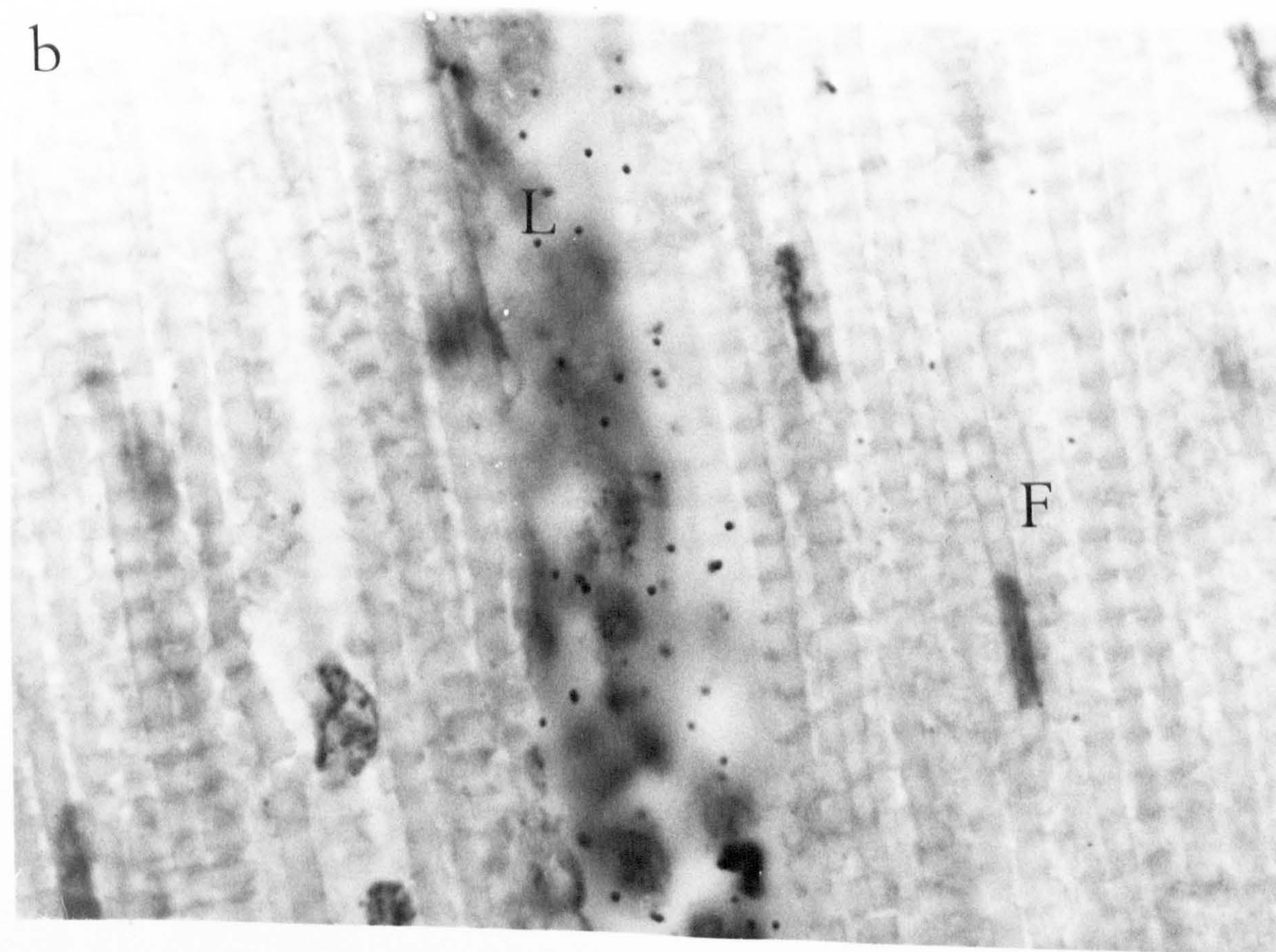
In the microfilaria Simpson and Laurence (1972) thought that the transport and efflux of solutes into the larva must be mediated by mechanisms located at the surface of the sheath and cuticle. As this study has demonstrated that the larva of Brugia patei selectively takes up metabolites from the external environment, it is reasonable to postulate that some type of active transport or facilitative diffusion mechanism exists at the surface of the larva similar to that known to exist at the body surface of other helminths (Pappas and Read, 1975).

According to Vincent, Ash and Frommes (1975), the cuticle of adult Brugia may be permeable to solutes. Consequently it should not be assumed that the incorporation of leucine-[H₃] into second and third stage larva of Brugia patei results from the ingestion of just host tissue mitochondria and other muscle elements. Because of its larger size and viability the third stage filarial larva is a potentially attractive model for pulse chase studies using a variety of labelled compounds.

PLATE 1

(a) Incorporation of lysine-[H₃] into Brugia patei parasitic in Aedes togoi. Injected at 92 hours pbm. F = flight muscle. L = larva.

(b) Incorporation of tryptophan-[H₃] into Brugia patei parasitic in Anopheles labranchiae atroparvus. Injected at 91.5 hours pbm. F = flight muscle. L = larva.



AUTORADIOGRAPHIC AND HISTOCHEMICAL OBSERVATIONS ON FLIGHT MUSCLE
PARASITISED BY BRUGIA PATEI

INTRODUCTION

1. Structure of muscle

In mosquitoes as in other insects, there are two kinds of muscle, smooth muscle associated with the visceral and reproductive organs, and skeletal muscle, which provides the power of locomotion (Snodgrass, 1935; Christophers, 1960; Wigglesworth, 1972). Both types of muscle consist of striated fibres. In mosquitoes, this striated muscle is organised into two types, thoracic flight muscle and tubular muscle, such as that present in the femur of the legs (Christophers, 1960).

The indirect flight muscles in the mosquito thorax are arranged in longitudinal and dorso-ventral groups (Beckett and MacDonald, 1968; 1970). Each muscle consists of a number of quite large fibres. The cytoplasm (sarcoplasm) of each muscle fibre contains rows of myofibrils (sarcostyles). The width of a myofibril is approximately 2.5 μm . Each myofibril contains arrays of alternating isotropic and anisotropic segments. The nuclei of the flight muscles are fusiform in shape and are randomly distributed. A characteristic feature of flight muscle is the presence of very large mitochondria, arranged in rows between the myofibrils. Glycogen is present in the spaces between the mitochondria, and also within the myofibrils. Tracheoles are closely associated with the flight muscle fibres. This brief outline of the structure of the mosquito thoracic flight muscle is based on the work of Christophers (1960) and Johnson and Rowley (1972). A general account of the ultrastructural features of insect flight muscle is given by Smith (1958). The biochemistry of insect flight muscle has been reviewed by Bailey (1975) and Sactor (1975).

2. Flight muscle parasitised by filaria

In the genus Brugia the parasite undergoes the first two larval moults within the indirect thoracic flight muscles of its mosquito host (Feng, 1936; Laurence and Pester, 1961; Schacher, 1962a). During this

intracellular phase of the parasite's existence within the muscles, a certain amount of attention has centred upon whether the parasite damages the muscle fibres which lie adjacent to it. The evidence to date indicates that the parasite causes minimal damage to the flight muscle during the initial phase of development, but that significant damage may occur when the third stage larva leaves the muscles and enters the haemocoel (Townson, 1970; Beckett, 1971; Kan and Ho, 1973).

During the early phase of filarial development within the flight muscle, it is possible that the parasite may exert some metabolic pressure on the muscle which lies adjacent to it, and which is not detectable by routine histological and ultrastructural examination. Consequently the incorporation of various radioactive metabolites by flight muscle which lies adjacent to the parasite was recorded and compared with flight muscle unassociated with the parasite but still within the same animal and with flight muscle from uninfected mosquitoes.

Observations were also made on the uptake of various metabolites into tubular muscle to determine whether significant differences in incorporation could be distinguished between flight muscle and tubular muscle. The latter does not appear to be a suitable site for filarial development.

Histochemical data on both types of muscle tissue, including flight muscle parasitised by Brugia patei, was collected in support of the autoradiographic findings.

3. Mechanical damage to flight muscle

The injection technique used to introduce labelled metabolites into the mosquito produced a puncture wound at the site of injection in the thorax, the glass capillary needles causing varying degrees of damage to the thoracic musculature. The damaged muscle appeared to be a site for focal melanisation, which would be an interesting model to compare with the host response elicited by the entry of Brugia patei into the flight muscles of Anopheles labranchiae atroparvus. The filarially induced host reaction is considered in the next section. Here the opportunity was taken to describe the mechanically damaged muscle in histopathological

terms, and to collect autoradiographic data on the uptake of labelled metabolites into the wounded areas.

RESULTS

1. Incorporation of nucleosides into flight muscle

In the flight muscles of Aedes togoi parasitised by Brugia patei an intense labelling with thymidine-[H₃] was present over the nuclei of some muscle fibres. This thymidine-[H₃] labelling of the muscle nuclei was commonly but not exclusively seen over nuclei in close proximity to filarial larvae. This pronounced nuclear labelling was not observed in thoracic muscle nuclei of parasitised mosquitoes that had been injected between 3-24 hours pbm, but was present in individuals that had been injected at 5 days pbm. Thymidine-[H₃] marking was also present over nuclei in the flight muscles of Anopheles labranchiae atroparvus injected with the isotope at 5 days pbm. As with Aedes togoi nuclear markings with tritiated thymidine was commonly seen in areas occupied by the parasites. In these 5 day mosquitoes many of the labelled nuclei near or adjacent to the worms were rounded and swollen in appearance and were not the typical fusiform shape.

In Aedes togoi parasitised by Brugia patei, uridine-[H₃] incorporation was also recorded over the flight muscles. The grain density was highest over the nucleoli. Cytoplasmic label was slight by comparison. In females that had been injected with the isotope at either 26.5 or 45 hours pbm, no significant degree of incorporation was recorded over the flight muscles (Table 6). A parasitised female of Anopheles labranchiae atroparvus also showed no muscle label that was significant relative to background radioactivity.

In parasitised Aedes togoi that had received an injection of uridine-[H₃] at 5, 6 or 8 days pbm pronounced nuclear marking was visible over nuclei adjacent to filarial larvae as well as over nuclei present in unparasitised regions of muscle. Many of the nuclei in these uridine-[H₃] injected animals were also swollen and rounded in shape. The hypertrophic nuclei were present only in the 5, 6 and 8 day animals. In the 8 day animals some of the hypertrophic nuclei were present in close proximity to the tissue spaces formerly occupied by the developing filarial larvae. Grain counting failed to distinguish differences in uptake of uridine-[H₃] by

Table 6 Incorporation of uridine-[H₃] into indirect thoracic flight muscles of (a) *Aedes togoi* (b) *Anopheles labranchiae atroparvus*: both species parasitised by *Brugia patei*

Time of injection (pbm)	Mean grain density $\bar{x} \pm$ standard error of mean		
	Muscle adjacent to parasite	Muscle away from parasite	Background
(a) <i>Aedes togoi</i>			
26.5 hours	2.00±0.34	1.86±0.25	2.03±0.11
45 hours	2.92±0.28	2.40±0.36	1.79±0.11
5 days	6.00±1.85	4.56±0.35	2.07±0.15
6 days	4.40±0.34	4.08±0.30	1.96±0.15
8 days		8.16±0.45	1.97±0.15
(b) <i>Anopheles labranchiae atroparvus</i>			
45 hours	7.0±1.02	8.28±0.45	4.70±0.20

One mosquito was sampled per time sequence.

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x})
per unit area of muscle = $\frac{\text{(total number grains counted)}}{25 \text{ unit areas/tissue}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{\text{(total number grains counted)}}{100 \text{ unit areas}}$

1 unit area = 64µm².

muscle fibres adjacent to filarial larvae compared with muscle fibres sampled from unparasitised regions of muscle. These results are summarised in Table 6.

2. Incorporation of carbohydrates into flight muscle

There was no significant incorporation of galactose-[H₃] and glucosamine-[H₃] into the flight muscles of Aedes togoi and Anopheles labranchiae atroparvus at 4 days pbm (Table 7). However glucose-[H₃] was incorporated into the flight muscles of Anopheles labranchiae atroparvus at 24, 48, 72 and 96 hours pbm. Furthermore in the 48-96 hour females the mean grain density of glucose-[H₃] label in flight muscle adjacent to abnormal larvae of Brugia patei was lower than that recorded for unparasitised muscle (Table 7).

In tubular muscle, the mean grain density of labelled carbohydrate did not differ significantly from that recorded for flight muscle. This applied to both mosquito species, and to all three isotopes used.

3. Incorporation of amino acids into the flight muscles

Incorporation of labelled amino acids into the indirect thoracic flight muscles was in general quite low (Tables 8, 9). Both mosquito hosts incorporated comparatively higher levels of the basic amino acid arginine, and in Anopheles labranchiae atroparvus the highest degree of incorporation was observed with the amino acid lysine, with a mean grain count of 27.93 ± 1.32 compared to a much lower mean grain count of 6.40 ± 1.11 in the flight muscles of Aedes togoi.

Although in Anopheles labranchiae atroparvus abnormal larvae of Brugia patei also incorporated comparatively high levels of lysine-[H₃], a comparison of amino acid incorporation in Tables 4 and 8 suggests that there is no direct relationship between uptake of labelled amino acid into the parasite and uptake into the flight muscle associated with the parasite. This view is strengthened by the finding that the mean grain density of labelled amino acid derived from muscle fibres adjacent to the parasite were not significantly different to those in sites away from the parasite (Tables 8, 9). Furthermore no significant differences

Table 7 Incorporation of [H₃]-carbohydrate into indirect thoracic flight muscle of (a) *Aedes togoi* (b) *Anopheles labranchiae atroparvus* when parasitised by *Brugia patei*

Isotope	Time of injection (hours pbm)	No mosquitoes used	Mean grain density $\bar{x} \pm$ standard error of mean		
			Muscle adjacent to worm	Muscle away from worm	Background
(a) <u>Aedes togoi</u>					
Galactose	92.5	1	(5)1.40 \pm 0.35	(5)1.40 \pm 0.21	1.03 \pm 0.10
Glucosamine	95	1	(5)2.80 \pm 0.76	(5)1.28 \pm 0.20	0.86 \pm 0.09
(b) <u>Anopheles labranchiae atroparvus</u>					
Glucose	24	3	(40)10.83 \pm 1.55	(75)12.64 \pm 0.64	1.96 \pm 0.18
	48	3	(15)4.85 \pm 1.0	(75)10.79 \pm 1.06	2.77 \pm 0.17
	72	2	(15)4.70 \pm 0.77	(50)12.16 \pm 1.43	1.60 \pm 0.14
	96	3	(25)5.35 \pm 0.88	(75)15.19 \pm 2.13	3.09 \pm 1.19
Galactose	24	1	(5)1.40 \pm 0.45	(25)4.04 \pm 3.90	3.39 \pm 0.22
	48	1	(15)5.00 \pm 1.26	(25)4.32 \pm 0.36	6.18 \pm 0.36
	72	1	(10)2.40 \pm 0.37	(25)7.56 \pm 0.96	5.53 \pm 0.25
	96	1	(10)2.60 \pm 0.63	(25)2.64 \pm 0.32	1.80 \pm 0.10
Galactose	92.5	1	(5)1.80 \pm 0.43	(5)4.00 \pm 1.46	1.03 \pm 0.09
Glucosamine	95	1	(5)2.00 \pm 0.40	(5)3.60 \pm 0.72	1.74 \pm 0.16

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x}) per unit area of muscle = $\frac{(\text{total number grains counted})}{\text{number mosquitoes used} \times \text{number unit areas sampled}}$

Figures in parentheses indicate number of unit areas sampled.

Mean grain density (\bar{x}) per unit area of background = $\frac{(\text{total number grains counted})}{\text{number mosquitoes used} \times 100 \text{ unit areas}}$

1 unit area = 64²μm .

in incorporation of phenylalanine-[H₃] were found between the flight muscles of infected and uninfected Anopheles labranchiae atroparvus and Aedes togoi (Table 9). An interesting exception to the above findings, and which deserves further study is that the mean grains counts over flight muscle samples from uninfected Anopheles labranchiae atroparvus that had been injected with DOPA-[H₃] were significantly greater ($p < 0.05$) than similar muscle from individuals infected with Brugia patei (Table 9). Of great interest here is the finding that DOPA-[H₃] incorporation by flight muscles of uninfected and filarially infected Aedes togoi was not significantly above background radioactivity (Table 9).

The incorporation of labelled amino acid into the tubular type of muscle was compared with that obtained for flight muscle. Grain counts taken from tubular muscle present in the legs did not differ significantly from those obtained for the flight muscle.

Grain counts over flight muscle were recorded from mosquitoes that had been injected with radioactive amino acid and then pulsed for different times before fixing in FA. It can be seen from Table 10 that extending the radioactive pulse resulted in only slight improvement in amino acid incorporation by the flight muscle (compare Table 3).

4. Histopathology and histochemistry of infected flight muscle

(a) Histopathology

In Aedes togoi and Anopheles labranchiae atroparvus infected with Brugia patei, larvae were rarely observed in histological sections of flight muscle sampled from mosquitoes killed earlier than 4 hours following the entry of the parasite into the host. Unfortunately despite the routine use of whole, serially sectioned mosquitoes, no sections were observed which showed the larvae fixed at the point of entry into the flight muscle. Following the entry of a larva into a muscle fibre it is enclosed within a tunnel which presumably results from the separation of myofibrils adjacent to the parasite. The space between the cuticle of the parasite and the myofibrils adjacent to it was usually slight, and in some cases, scarcely detectable (Plate 1). From the second day

Table 8 Incorporation of [H₃]-amino acids into indirect thoracic flight muscle of (a) *Aedes togoi* (b) *Anopheles labranchiae atroparvus*: both species parasitised by *Aedes togoi*

Isotope	Time of injection (hours pbm)	Mean grain density $\bar{x} \pm$ standard error of mean		
		Muscle sampled adjacent to parasite	Muscle sampled away from parasite	Background
(a) <u>Aedes togoi</u>				
Histidine	96	2.8 \pm 0.86	2.20 \pm 0.52	2.35 \pm 0.13
Proline	120	3.00 \pm 0.90	5.20 \pm 1.19	0.83 \pm 0.09
Arginine	97	11.20 \pm 1.50	13.80 \pm 5.04	3.28 \pm 0.18
Lysine	92	6.40 \pm 1.11	4.80 \pm 0.82	1.08 \pm 0.25
Methionine	95	5.40 \pm 1.08	6.20 \pm 0.65	1.99 \pm 0.14
Cystine	99	8.00 \pm 0.8	6.00 \pm 0.97	5.21 \pm 0.23
Iso-leucine	91	6.25 \pm 2.25	6.20 \pm 1.29	<1
Valine	90.5	3.20 \pm 0.91	3.68 \pm 0.28	0.72 \pm 0.10
Tryptophan	91.5	5.00 \pm 1.01	3.52 \pm 0.45	0.64 \pm 0.07
Hydroxytryptophan	98	5.40 \pm 0.86	5.10 \pm 0.16	4.42 \pm 0.18
(b) <u>Anopheles labranchiae atroparvus</u>				
Histidine	96	4.20 \pm 0.86	2.60 \pm 0.45	2.76 \pm 0.12
Proline	120	7.20 \pm 1.42	8.60 \pm 0.45	2.02 \pm 0.13
Arginine	97	8.40 \pm 0.56	9.20 \pm 1.30	2.56 \pm 0.13
Lysine	92	27.93 \pm 1.32	29.80 \pm 1.09	<1
Methionine	95	4.60 \pm 0.60	6.68 \pm 0.20	0.82 \pm 0.08
Cystine	99	7.60 \pm 1.34	6.00 \pm 0.80	3.80 \pm 0.25
Iso-leucine	91	5.16 \pm 1.17	6.40 \pm 0.91	<1
Valine	90.5	2.88 \pm 0.37	2.20 \pm 0.64	<1
Tryptophan	91.5	2.36 \pm 0.30	2.48 \pm 0.34	0.83 \pm 0.03
Hydroxytryptophan	98	4.40 \pm 0.35	5.96 \pm 0.48	4.41 \pm 0.15

One mosquito of each species was used for each amino acid.

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x})
per unit area of muscle = $\frac{(\text{total number grains counted})}{5 \text{ unit areas}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{(\text{total number grains counted})}{100 \text{ unit areas}}$

1 unit area = 64 μm^2 .

of infection onwards, the growth of the larva (Feng, 1936) probably exerts pressure on the surrounding flight muscles, but not severe enough to induce extensive damage to the myofibrils. The tunnel enlarges to accommodate the growing worm. In CHP sections phloxinophil debris was present at either end of the parasite from the second day of the infection onwards. These findings are in agreement with the earlier observations of Beckett and other workers (Beckett and Boothroyd, 1969; 1970; Beckett, 1971).

Apart from the presence of a host encapsulation substance around the parasite when the latter was parasitic in Anopheles labranchiae atroparvus (Part 5), no clear host associated differences in parasitised flight muscle was detected between the two mosquito species. Furthermore, liquefactive necrosis of the flight muscle that could unequivocally be attributable to the presence of the parasite was not observed. There were individuals of both species where parasitised flight muscle showed varying degrees of disruption, ranging from a loss of the normal parallel array of the myofibrils to liquefactive necrosis of areas of flight muscle. Plate 1(a) shows the appearance of disrupted muscle myofibrils near to but not adjacent to a larval Brugia patei. Disrupted flight muscle tissue was seen frequently in unparasitised mosquitoes.

After an infective feed, some individual Anopheles labranchiae atroparvus presumably ingested greater than usual numbers of microfilariae of Brugia patei. These mosquitoes were moribund, resting mostly on the floor of the cage and showing little flight activity when disturbed. The mosquitoes were fixed in FA at 19 hours pbm and examined histologically. Estimating accurately the total number of filarial larvae present in the thoracic flight muscles was difficult. However it was clear that some individuals had a worm burden in excess of 60 in the flight muscles. Even in these comparatively heavily infected individuals damage to the flight muscles was slight, and development of the larvae still apparently normal at this stage.

(b) Histochemistry

In a further attempt to identify parasite induced flight muscle damage, histochemical observations were recorded on uninfected and infected

Table 9 Incorporation of [¹⁴C] phenolic amino acids into indirect thoracic flight muscle parasitised by *Brugia patei*, including a comparison with flight muscle from uninfected animals

Isotope	Time of injection (hours pbm)	No mosquitoes used	Mean grain density $\bar{x} \pm$ standard error of mean			
			Muscle \bar{x}	Background \bar{x}	Uninfected muscle \bar{x}	Background \bar{x}
(a) <u>Aedes togoi</u>						
Phenylalanine	93	1	6.10 \pm 4.80	<1	4.80 \pm 0.36	<1
DOPA	93	1	0.60 \pm 0.75	4.40 \pm 0.49	0.68 \pm 0.14	0.52 \pm 0.05
(b) <u>Anopheles labranchiae atroparvus</u>						
Phenylalanine	24	1	3.28 \pm 0.50	1.55 \pm 0.12	3.04 \pm 0.33	1.19 \pm 0.09
	48	1	6.12 \pm 0.54	3.33 \pm 0.29	3.20 \pm 0.51	1.16 \pm 0.11
	72	1	5.36 \pm 0.66	3.11 \pm 0.19	7.16 \pm 0.63	<1
	96	1	4.40 \pm 0.69	1.79 \pm 0.89	4.76 \pm 0.49	1.09 \pm 0.09
Tyrosine	24	2	4.29 \pm 0.40	1.72 \pm 0.27		
	48	2	4.64 \pm 0.34	1.10 \pm 0.15		
	72	2	7.14 \pm 0.35	2.83 \pm 0.87		
	96	1	0.84 \pm 0.10	<1		
DOPA	93	2	4.92 \pm 0.36	<1	10.16 \pm 0.48	<1
DOPA	24	3	5.64 \pm 0.67	1.42 \pm 0.33		
	48	2	3.90 \pm 0.40	1.09 \pm 0.16		
	72	3	3.76 \pm 0.44	1.03 \pm 0.10		
	96	2	5.72 \pm 0.39	<1		

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x}) per unit area of muscle = $\frac{\text{(total number grains counted)}}{\text{number mosquitoes used} \times 25 \text{ unit areas/tissue}}$

Mean grain density (\bar{x}) per unit area of background = $\frac{\text{(total number grains counted)}}{\text{number mosquitoes used} \times 100 \text{ unit areas}}$

1 unit area = 64 μm^2 .

flight muscle.

With methyl green pyronin the muscle nuclei stained green, the nucleoli bright red. The sarcoplasm was virtually unstained with pyronin. The latter finding agreed with the previously recorded low degree of uridine-[H₃] label over the muscle sarcoplasm. In the absence of obvious morphological damage to the flight muscles, no distinction in nucleic acid staining pattern was observed between parasitised and non parasitised muscle.

The distribution of flight muscle glycogen in uninfected and filarially infected individuals of Aedes togoi and Anopheles labranchiae atroparvus was studied by the application of the PAS, Sodium bismuthate Schiff, Sodium bisulphite toluidine blue and Best's Carmine methods. With these techniques stainable material that was extractable by pre-treatment with amylase was considered to be glycogen. In both mosquito species, glycogen was demonstrable in flight muscle and tubular muscle of individuals fixed soon after eclosion and at various times up to 14 days pbm. In the flight muscles the polymer was present between the myofibrils and as thin bands along them. Some of the parasitised flight muscle fibres contained areas around the parasites which were depleted of glycogen (Plate 2). Yet even in unparasitised flight muscle from the same animal and in muscle from uninfected mosquitoes there were marked differences in the distribution of glycogen between the fibres.

The protein histochemical techniques failed to detect any difference in staining between flight muscle adjacent to the parasite compared to that from uninfected flight muscle. There was also no evidence of any non specific esterase, acid phosphatase and β -glucosaminidase activity in unparasitised mosquito flight muscles.

Considering these histochemical findings in relation to the autoradiographic data, it is concluded that the parasite causes at most only a minimum degree of damage to the host flight muscles during the period up to the first moult.

Table 10 The effect of changing the duration of the
radioactive pulse on [113]-amino acid
incorporation into thoracic flight muscle

Isotope	Time of injection (hours pbm)	Duration of pulse (mins)	Mean grain density \bar{x} ± error of mean	
			Muscle	Background
(a) <u>Aedes togoi</u>				
Histidine	96	30	2.20±0.50	2.35±0.13
		120	7.68±0.55	2.61±0.12
Proline	120	30	4.60±0.55	0.83±0.09
		120	7.20±0.52	2.97±0.13
Tyrosine	117	30	5.36±0.49	2.83±0.74
		120	4.00±0.32	2.78±0.19
Leucine	96	10	<1	<1
		20	2.40±0.45	0.70±0.15
		30	3.60±1.95	2.71±0.31
		120	9.68±0.56	2.70±0.14
(b) <u>Anopheles labranchiae atroparvus</u>				
Tyrosine	117	30	4.40±0.66	2.23±0.10
		120	6.60±0.27	2.24±0.10
		240	3.52±0.27	2.00±0.15
		360	5.00±0.30	2.76±0.13

One mosquito of each species was used per time interval.

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x})
per unit area of muscle = $\frac{\text{(total number grains counted)}}{5 \text{ unit areas}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{\text{(total number grains counted)}}{100 \text{ unit areas}}$

1 unit area = 64 μm^2 .

5. Histopathology and autoradiography of mechanically induced flight muscle damage

The punctures made by the glass capillary needles used to inject labelled metabolites were generally discrete and correspondingly damage to the thoracic musculature was quite localised. In serial sections the site of injury was recognised as a circular hole in the muscle (Plates 3, 4). Around the hole there was a localised disruption of muscle elements. The latter was sometimes accompanied by focal necrosis and melanisation of muscle tissue adjacent to the hole (Plate 5). This mechanically induced muscle necrosis could occur quite quickly after the injection. For example in individuals which had been fixed for histology after having blood removed via needle puncture through the thorax, muscle necrosis was recorded in individuals that had been fixed a few minutes after the puncture. Some examples of damaged flight muscle tissue are illustrated in Plates 3 to 6.

Another interesting aspect of the injection technique was that occasionally when performing the injection, the tip of the needle broke and was left behind when the pipette was withdrawn. It was not uncommon to find broken fragments of the integument including hairs and scales that had been introduced deep into the thoracic musculature. When this occurred both glass and tissue fragments became melanised. The melanin deposited around these implants incorporated a number of known melanin precursors as well as glucose-[H₃] (Plate 4). In fact of all the labelled metabolites which were injected into the mosquito via a thoracic puncture, only those which were capable of being incorporated into melanin (Part 5) showed a significantly higher mean grain density over melanin plaques and damaged muscle around a wound, compared with undamaged muscle (Table 11).

The DOPA decarboxylase inhibitor α MDH (Part 5) when injected into the thorax of Anopheles labranchiae atroparvus parasitised by Brugia patei did not prevent the subsequent formation of melanin plaques around the wound.

Using DOPA-[H₃], cells which were heavily labelled with this compound were observed in the vicinity of the damaged muscle. These cells were

scattered among elements of damaged muscle (Plate 6). The possibility that the small cells represent intensely labelled haemocytes is considered in Part 5.

Table 11 Incorporation of [¹⁴C]-phenolic and basic amino acid into tissue around puncture wounds in the thorax of *Anopheles labranchiae atroparvus*

Isotope	Time of injection (hours pbm)	Mean grain density $\bar{x} \pm$ standard error of mean			
		Muscle around wound	Undamaged muscle	Melanin around wound	Background
(a) Infected with <u>Brugia patei</u>					
Phenylalanine	24	104.00 \pm 8.36	2.04 \pm 2.48	119.20 \pm 12.15	<2
Tyrosine	48	54.40 \pm 4.40	6.00 \pm 1.44	>100	<2
DOPA	72	35.20 \pm 2.08	3.00 \pm 0.84	>100	<2
Arginine	24	13.24 \pm 0.73	6.08 \pm 0.45	No melanin observed	<2
(b) Uninfected					
Phenylalanine	24	46.24 \pm 2.68	2.40 \pm 0.64	No melanin observed	<2

One mosquito was sampled at each time sequence.

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density \bar{x} per unit area of tissue = $\frac{\text{(total number of grains)}}{5 \text{ unit areas}}$

Mean grain density \bar{x} per unit area of background = $\frac{\text{(total number of grains)}}{100 \text{ unit areas}}$

1. unit area = 64 μm^2 .

DISCUSSION

The incorporation of thymidine-[H₃] into the nuclei of parasitised muscle fibres of both mosquito hosts was not observed during the first 4 days of filarial development, but at day 5 some of the muscle nuclei were intensely labelled with thymidine-[H₃]. The labelled nuclei were commonly associated with the site of larval development, but as control, uninfected mosquitoes were not taken at this time, it cannot be demonstrated that the nuclear labelling results from the presence of the parasite. It is known that thymidine-[H₃] can become incorporated into cells which do not necessarily undergo mitosis (Fox, 1970; Pelc, 1964; 1972). Omar and Gwadz (1974) recorded a similar pattern of thymidine-[H₃] incorporation into flight muscle of Aedes aegypti parasitised by developing larvae of Brugia pahangi also commenting on the abnormal morphology of the nuclei of parasitised muscle.

In Aedes togoi, flight muscle labelling with uridine-[H₃] was present only in muscle fibres of individuals that had been fixed between 5-8 days following the entry of Brugia patei. Further studies are needed to determine whether this intense nuclear labelling of flight muscle with radioactive nucleoside is also a feature of uninfected Aedes togoi and Anopheles labranchiae atroparvus.

Beckett (1971) reported that in flight muscles of Aedes aegypti and Mansonia uniformis infected with Brugia malayi, enlarged (hypertrophic) muscle nuclei were present in some fibres as early as 48 hours after the infecting blood meal. In Aedes togoi, parasitised by Brugia patei hypertrophic muscle nuclei were not observed until at least 5 days pbm. In Anopheles labranchiae atroparvus enlarged muscle nuclei were seen in one individual that had been injected with thymidine-[H₃] at 5 days pbm. In contrast, in routine CHP-stained sections, hypertrophic muscle nuclei were not observed even in females that had been fixed at 10 days pbm. These nuclear abnormalities were not observed in uninfected control mosquitoes.

The appearance of these enlarged nuclei in parasitised mosquitoes

coincides in Aedes togoi with the moult of Brugia patei to the second larval stage, sometime between the period 4-5 days pbm. This suggests that the presence of nuclear abnormalities and nucleoside incorporation into muscle nuclei at 5 or more days pbm is connected to the transformation of the parasite into a form which is capable of ingesting particular material of host muscle origin (see Beckett and Boothroyd, 1970; Townson, 1970; Laurence and Simpson, 1971). Omar and Gwadz (1974) associated the thymidine- $[H_3]$ marking of the flight nuclei with the presence of the mature larva. The presence of labelled muscle nuclei in the refractory mosquito where development of the parasite does not normally progress beyond the first larval stage represents a finding at variance with the above suggestion. No mature third stage larvae were recorded in Anopheles labranchiae atroparvus. Possibly these nuclear abnormalities represent a stress response which is related more to the age of the infection in the muscle and is independent of the state of larval development reached by the worm. Further work is needed to clarify this point.

It is generally recognised that an important histological indicator of necrosis is the cell nucleus. If the insult to the cell is sufficiently severe, necrosis will begin. One of the earliest signs of degenerative change is recognised as a loss of the normal distribution of chromatin. The nucleus becomes shrunken, irregular in outline and more basophilic. This condition is termed pycnosis. Other nuclear changes that occur in damaged cells are swelling of the nucleus and then lysis (Karyolysis), and fragmentation (Karyorhexis). A nucleus which is undergoing Karyolysis will progressively lose its affinity for basic dyes. Degenerative nuclear changes in invertebrates appear to follow the same sequential pattern as those which occur in the vertebrates (Sparks, 1975). Brugia patei developing abnormally in the refractory strains of Aedes aegypti provide a good example of Karyolytic change. The larvae lyse and disappear without invoking a melanin encapsulation response (see Laurence, 1970).

In parasitised Aedes togoi and Anopheles labranchiae atroparvus the swollen muscle nuclei may represent a subacute stress response, which in the absence of recovery will progress through Karyolysis to ultimate necrosis of the muscle cell.

In this study necrosis of flight muscle parasitised by first stage larvae of Brugia patei was very rare. Esslinger (1962) recorded that moribund Anopheles quadrimaculatus infected with Brugia pahangi and examined at 18 hours pbm, showed necrosis, including liquefaction of the infected flight muscle tissue. In this laboratory, Anopheles labranchiae atroparvus heavily parasitised by Brugia patei, and examined at 19 hours pbm, failed to show extensive muscle damage. These contrasting results indicate the variation in host response that exists within the same genus of mosquito.

Recently, Kan and Ho (1973) described ultrastructural changes in the flight muscles of Aedes togoi which appear to be associated with the presence of developing Brugia patei. These changes included mitochondrial disorganisation and a reduction of interfibrillary glycogen in parasitised muscle. The glycogen loss was recorded as occurring as early as 2 days pbm. In Anopheles labranchiae atroparvus parasitised by Brugia patei, glucose-[H₃] is probably incorporated into glycogen within a 30 minute pulse period. The glucose-[H₃] silver grain distribution over the flight muscles matched closely that obtained by histochemical techniques used to demonstrate glycogen, even to the extent that the artifact of glycogen streaming was easily visible in the autoradiographs. This streaming artifact (polarisation) is seen when glycogen is displaced to one side of the cell as a result of unsatisfactory fixation (Pearse, 1968; Hopwood, 1969). Streaming artifact was absent in freeze dried vapour fixed sections.

It is tempting to explain the relatively lower mean grain density of glucose-[H₃] labelled flight muscle adjacent to the parasite as a condition which arises from the parasite's presence. At 24 hours pbm flight muscle sampled adjacent to the parasite was not significantly different from that sampled from unparasitised muscle. Yet at 48-96 hours when the larvae are undergoing rapid differentiation, the glucose-[H₃] label around the worms was significantly lower than that that from unparasitised muscle (Table 7).

Glycogen staining around the parasite was reduced or absent in histochemically stained sections. Glycogen was also depleted or absent around obviously damaged flight muscle such as that adjacent to the

hole made by a glass capillary injection pipette. Whether local glycogen depletion is a function of slightly damaged flight muscle remains to be determined. It is interesting that recent work on the biochemistry of the microfilariae of Wuchereria bancrofti has shown that the enzyme amylase (EC 3.2.1.1.) is present (Govindwar, Gawande and Harinath, 1974). An item for the protocol of future studies could be to determine to what extent glycogen streaming artifact in FA fixed material contributes to the histological picture described above. A study on the glycogen autoradiography and histochemistry of filarially infected mosquito flight muscle but using freeze dried vapour fixed specimens might resolve the problem.

If the muscle adjacent to the filarial larvae suffered any appreciable damage to the structural proteins it is possible that this would be reflected by differences in labelled amino acid incorporation between unparasitised and parasitised muscle. No significant differences in incorporation were recorded between the two classes of muscle even when 13 different amino acid markers had been assessed. The single exception was when DOPA-[H₃] was used. In Anopheles labranchiae atroparvus the mean grain density over flight muscle from uninfected individuals was much higher than that recorded over parasitised females. In the infected females no differences in DOPA-[H₃] uptake was found between muscle sampled adjacent to a filarial larva compared to that from an unparasitised region of flight muscle.

In severely damaged flight muscle the mean grain density of arginine-[H₃], phenylalanine-[H₃], tyrosine-[H₃] and DOPA-[H₃] was very high compared to the undamaged flight muscle (Table 11). All of these compounds except arginine are known melanin intermediates (Part 5), and all including arginine-[H₃], galactose-[H₃] and glucose-[H₃] became incorporated into the melanin pigment arising around Brugia pateri in Anopheles labranchiae atroparvus, and around mechanically damaged muscle in this species (Part 5).

It seems more probable that these isotopes are incorporated into melanin at the site of tissue damage rather than as part of any muscle reparative process. The incorporation of arginine-[H₃] into severely damaged flight muscle represents the only likely candidate for repair of damaged muscle.

Mosquito flight muscle stains strongly for basic protein including arginine.

The tritium labelled amino acid studies failed to provide autoradiographic evidence of damage or the repair of muscle damage resulting from the presence of the parasite in the tissue during normal development in Aedes togoi.

The host reaction of Anopheles labranchiae atroparvus is considered next.

PLATE 2

Brugia patei in Anopheles labranchiae atroparvus, showing depletion of glycogen in flight muscle adjacent to the larva. Fixed in FA at 4 days pbm and stained with Sodium bisulphite toluidine blue. F = glycogen rich flight muscle. D = glycogen depleted myofibrils adjacent to larva (L).

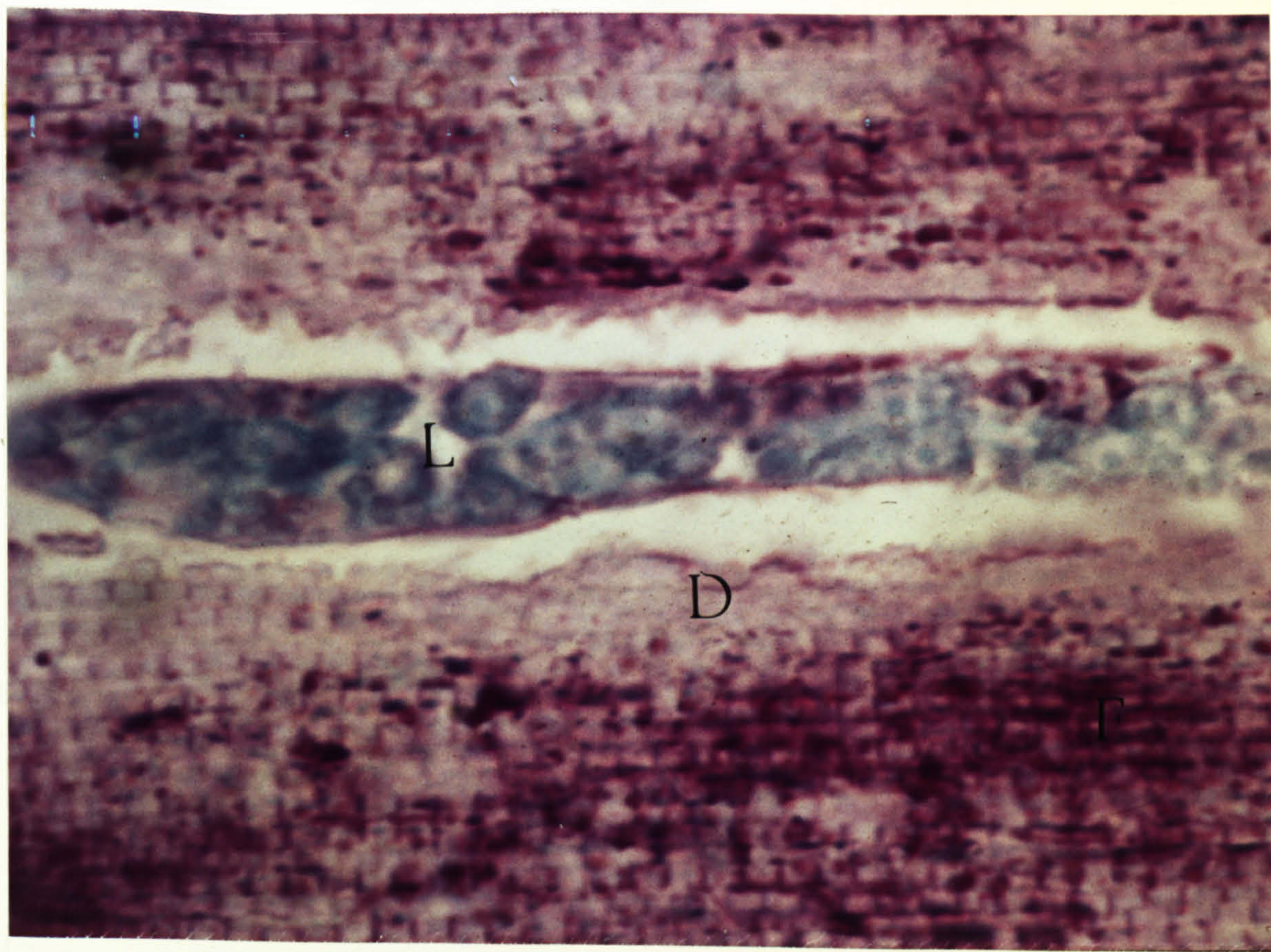


PLATE 3

Flight muscle of Anopheles labranchiae atroparvus
showing loss of glycogenⁱⁿ muscle fibres damaged by
capillary injection technique. Injected with saline at
4 days pbm, fixed in FA and stained with PAS. M = melanin.
F = undamaged flight muscle fibres. D = glycogen depleted
and damaged flight muscle fibre.

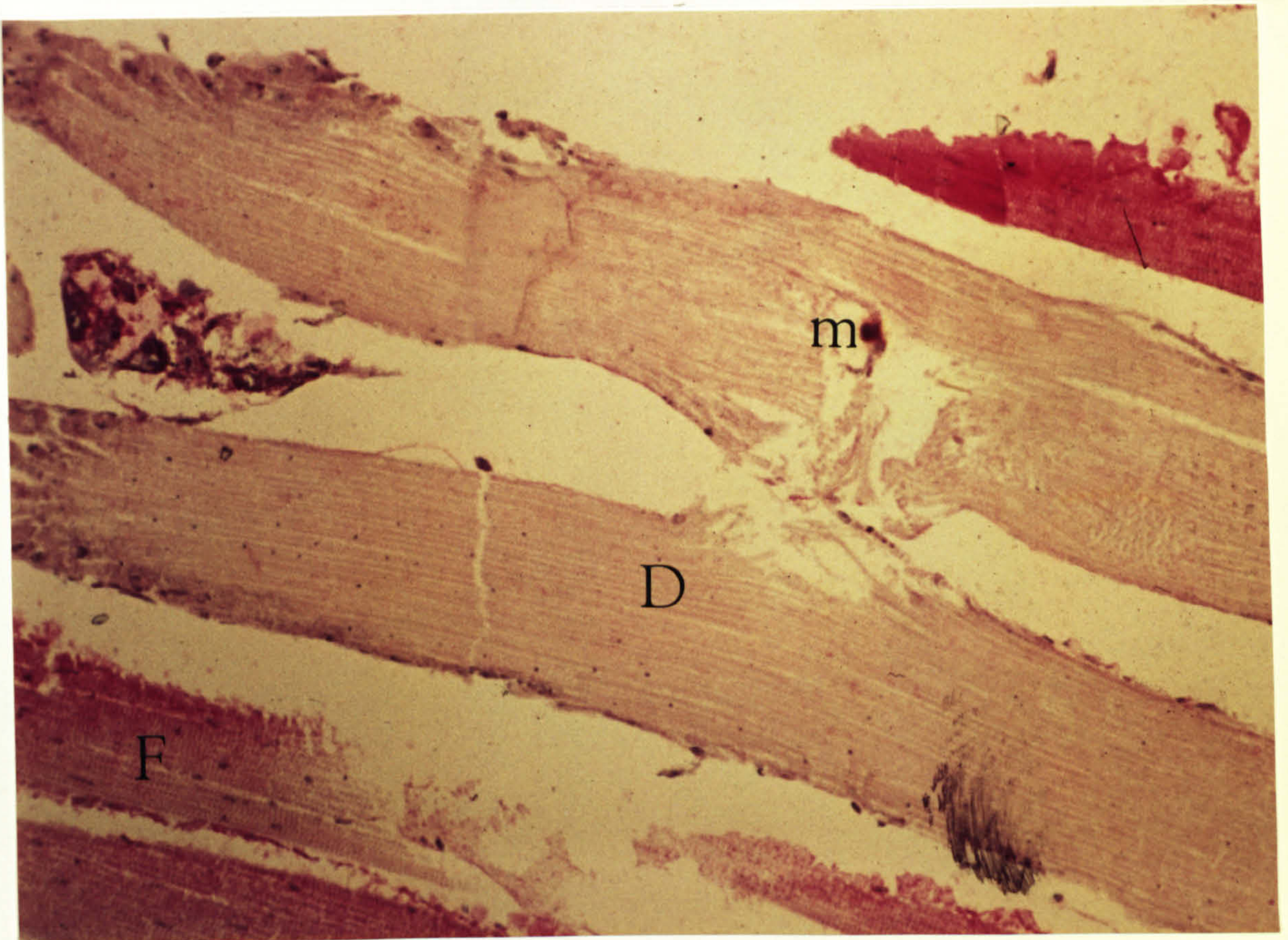
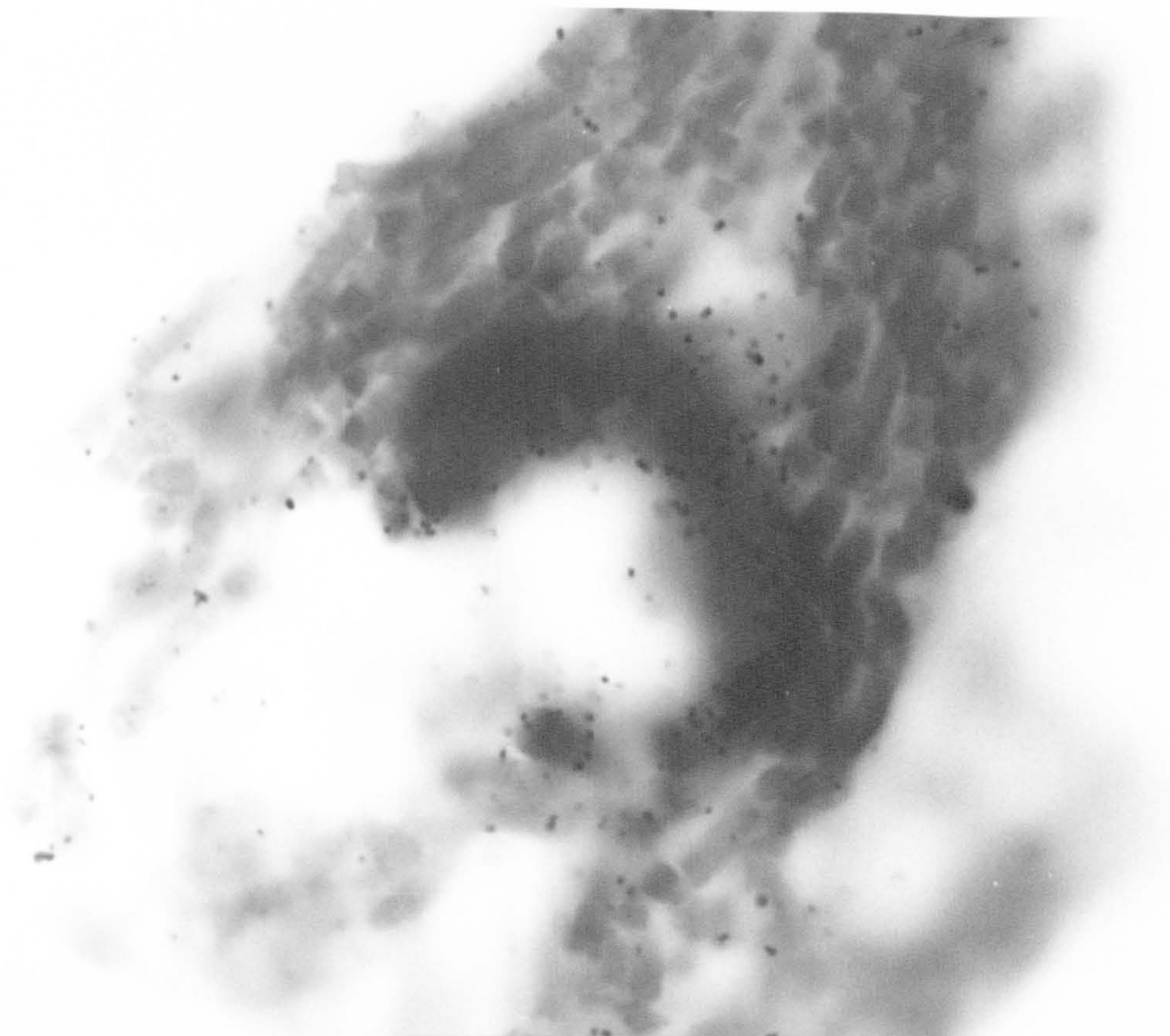


Fig. 1. Histological section of muscle tissue. In (a) the fibers are stained with eosin, in (b) on the muscle.

PLATE 4

Flight muscle of Anopheles labranchiae atroparvus showing incorporation of glucose-[H₃] into melanin formed at site of capillary injection. Time of injection was 24 hours pbm. F = flight muscle. M = melanin pigment. In (a) the focus is on the silver grains, in (b) on the muscle.

a



b

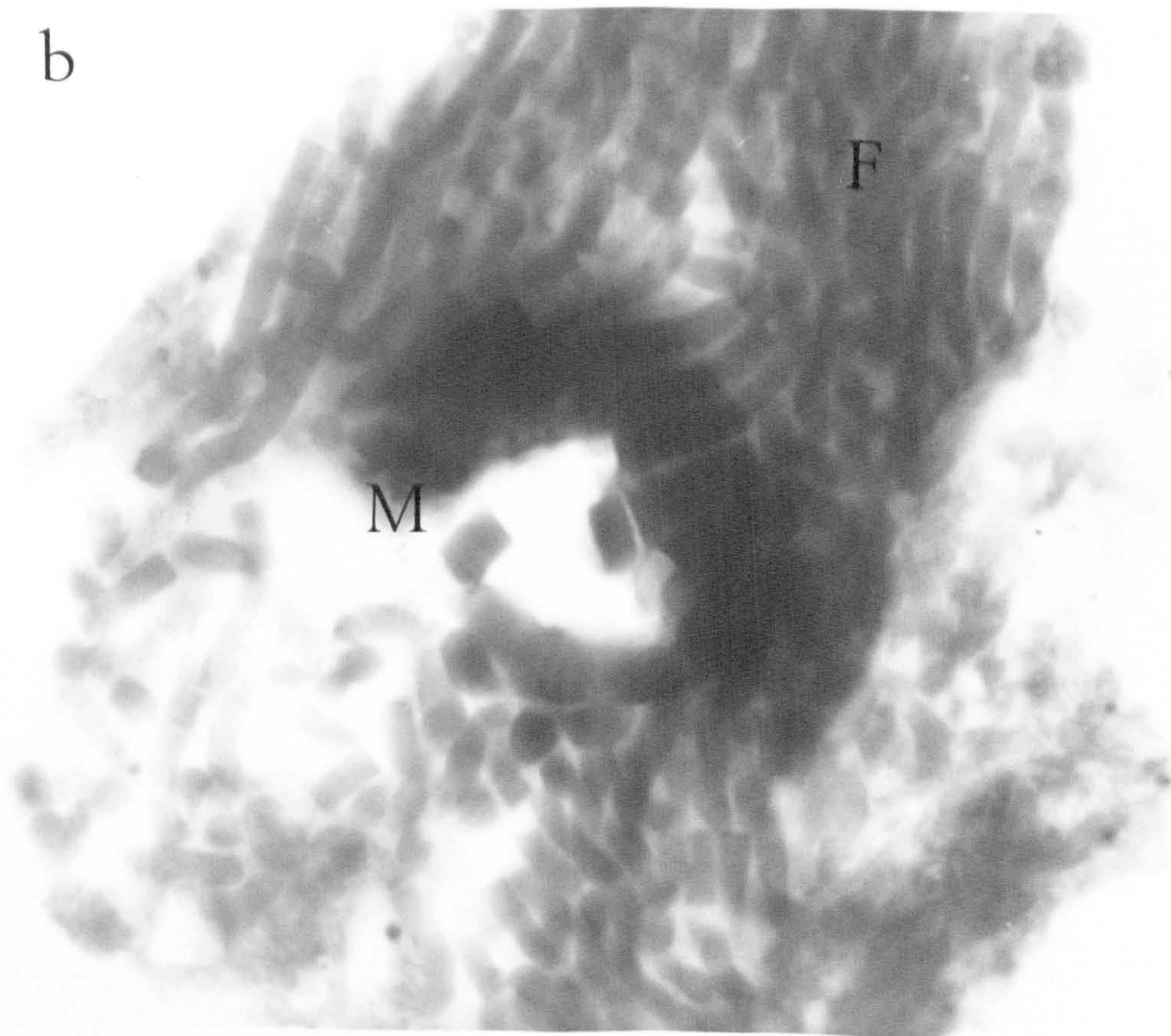


PLATE 5

(a, b) Flight muscle of Anopheles labranchiae atroparvus parasitised by Brugia patei. Injected with saline at 4 days pbm, fixed in FA and stained with PAS. M = melanin. F = undamaged flight muscle. D = glycogen depleted and severely damaged flight muscle fibre. Note the presence of the larva (L) in undamaged muscle which shows no loss of glycogen. ev = melanised excretory vesicle of larva.

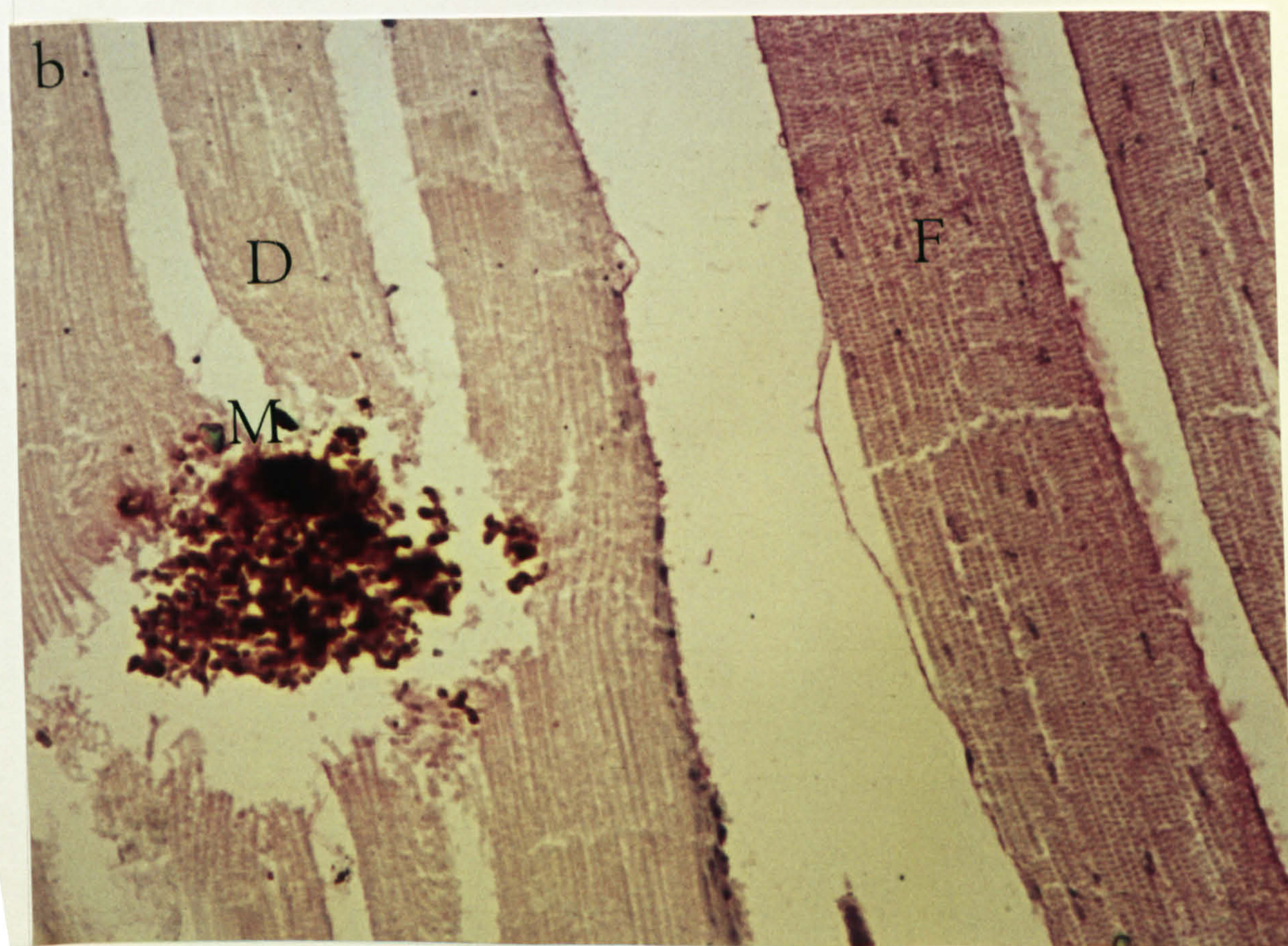
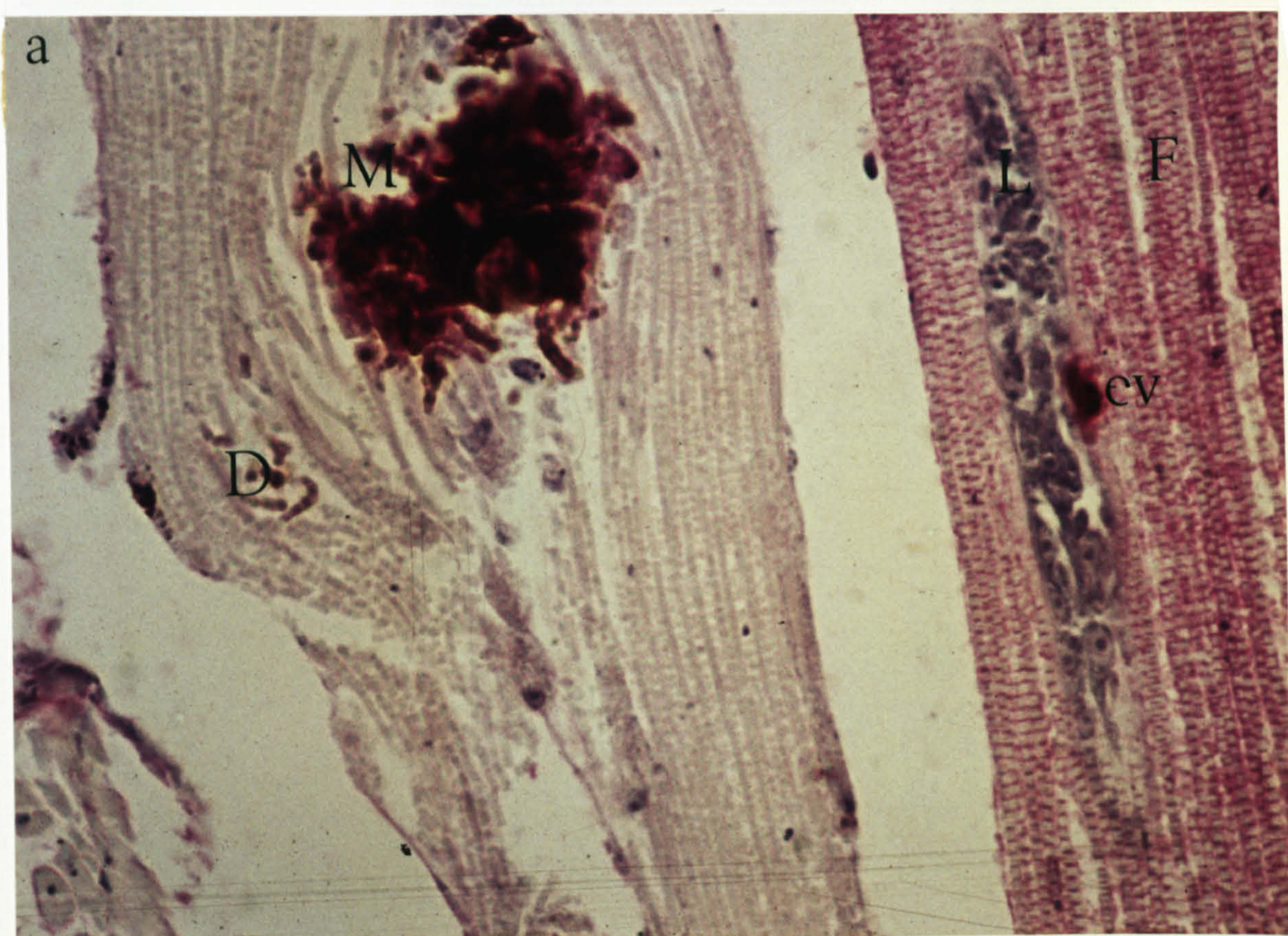
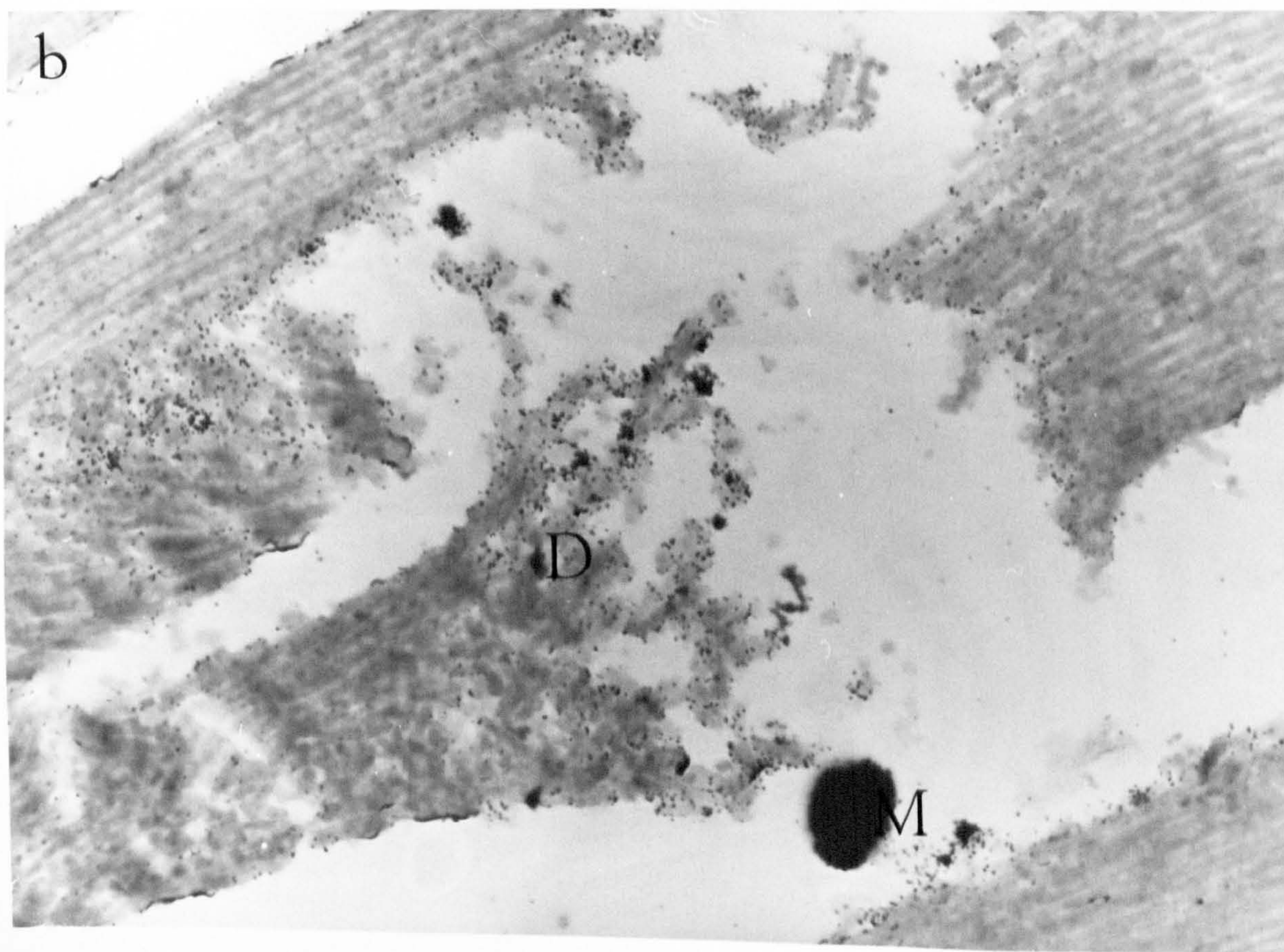
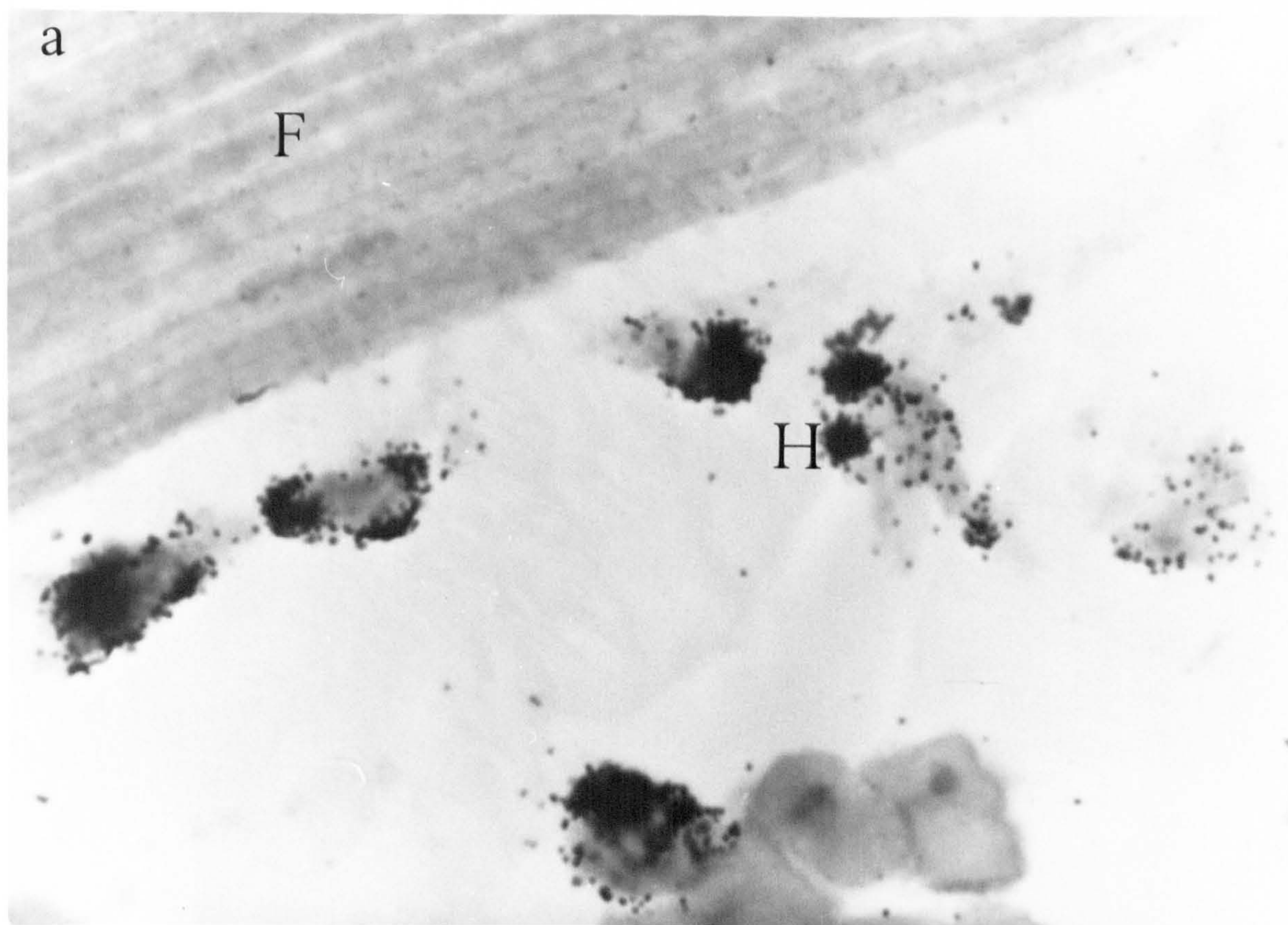


PLATE 6

(a, b) Flight muscle of Anopheles labranchiae atroparvus, parasitised by Brugia patei. Injected with DOPA-H3 at 24 hours pbm. H = haemocytes, F = flight muscle, D = damaged flight muscle, M = blobs of melanin. In (a) the haemocytes are adjacent to either fat body or muscle. In (b) intense labelling with DOPA-H3 is present at the site of the injection.



PART 5AUTORADIOGRAPHIC AND HISTOCHEMICAL FEATURES OF THE HOST RESPONSE OF
ANOPHELES LABRANCHIAE ATROPARVUS TO BRUGIA PATEIINTRODUCTION

In the preceding sections it was reported that a variety of tritium labelled metabolites became incorporated into the parasite and flight muscle tissue. In this section a report is given of the incorporation of these labelled metabolites into the host reaction that develops around Brugia patei following the entry of the parasite into the refractory mosquito, Anopheles labranchiae atroparvus (Laurence, 1970; Oothuman, Simpson and Laurence, 1974).

Some attention is given to the tissues in the mosquito which may be responsible, or associated with the production of the host response. In this section, the tissues considered are the blood cells, heart and associated pericardial cells, oenocytes and fat body cells. The reasons for considering these cell types as possible candidates involved in the production of the host reaction are given below. Initially, it is useful to say a little about the host reaction as a defence response in insects, and what particular form it takes in mosquitoes.

1. Host reaction

The observed immune reactions of insects against internal metazoan parasites fall into two currently recognised categories. In the first, there is an aggregation of host haemocytes to form a capsule around the parasite or foreign body (Salt, 1963, 1968, 1970; Whitcomb, Shapiro and Granadas, 1974). This process is commonly, but not exclusively accompanied by the deposition of a brown pigment onto the surface of the parasite (Nappi, 1975).

In the second category, can be grouped those insects in which the deposition of brown pigment onto the surface of the parasite seems to occur without the close intervention of haemocytes (Whitcomb, Shapiro and Granadas, 1974; Nappi, 1975). This type of response is classified

as humoral in origin. In both types of host response the brown pigment deposited on the surface of the parasite is assumed to be melanin (Poinar, 1969; Salt, 1970). In the case of the encapsulation of filarial nematodes in mosquitoes, the term melanisation has replaced the older term chitinisation (see Poinar, 1969). Despite the use of the term melanisation to describe the process of pigment deposition on the parasite surface, hard core evidence for the precise identification of the pigment as melanin is lacking. Presumptive evidence for the existence of melanin at the surface of encapsulated parasites is derived from two principle sources. The first is the use of histochemical methods to identify the pigment (Poinar, Leutenegger and Gotz, 1968; Poinar and Leutenegger, 1971; Gotz and Vey, 1974); the second is the use of chemical inhibitors to suppress or block the enzymes believed to be involved in the process of melanogenesis (Salt, 1956; Brewer and Vinson, 1971; Nappi, 1973). The specificity of the methods used in either case, will not stand critical examination. A major difficulty here is the realisation that there are a number of melanins, known to exist in nature, none of which can be described in precise chemical terms (Thomsen, 1962; Nicolaus, 1968). The latter restricts the term melanin to pigments derived from the enzymatic oxidation of tyrosine and to closely related compounds, including DOPA. Some melanins probably consist of a polymer of 5,6-dihydroxyindole units which may not only contain additional quinone derivatives, but also be conjugated to protein (Thomsen, 1962). In insects, indole melanin has been demonstrated in the black puparium of a mutant of Lucilia cuprina (Hackman, 1967) and from the cuticle of puparia and adults of the fleshfly, Sarcophaga bullata (Fogal and Fraenkel, 1969). The melanin of the fruit fly, Drosophila melanogaster is mainly an indole melanin (Fattorusso, Piatelli and Nicolaus, 1965). The work of Bins et al (1970) in a study of synthetic melanin formation showed that indole subunits were an apparently principle feature of melanin derived from dopamine. Clearly indole melanins have been proved by chemical analysis to exist in cuticular extracts of some larval and adult flies. The inference is that similar material is deposited around metazoan parasites and foreign bodies such as implants of araldite or glass beads.

Histochemical tests for the identification of melanins are not particularly specific and rely on the ability of melanins to reduce

ammoniacal silver nitrate, together with an insolubility in organic solvents, and the slow decolourisation by oxidising agents such as chromic acid, sulphuric acid, per-acids etc (Barka and Anderson, 1963; Lillie, 1965). In mammalian systems, the formation of melanin from tyrosine is dependant on the activity of a phenol oxidase (polyphenol oxidase, phenolase, dopa oxidase, catechol oxidase, tyrosinase). Histochemical methods exist for the localisation of this enzyme in tissues (Pearse, 1970; Chayen, Bitensky and Butcher, 1973). In his review of melanin involvement in host parasite interactions, Lipke (1975) has commented on the reluctance of cytologists interested in melanogenesis to exploit these histochemical techniques. However, this reluctance probably springs from an awareness of the non specificity of the term 'tyrosinase' when applied to the arthropods. The latter, unlike its mammalian counterpart, is only one of a series of enzymes involved in the conversion of soft cuticle into a hard sclerotised state (Hackman, 1974; Neville, 1975).

In the case of compounds used to inhibit the deposition of melanin on parasites in insects, Salt (1956) blocked the deposition of the pigment onto eggs of the parasite Nemeritis canescens parasitic in Carausius morosus by injecting the host with phenylthiourea (PTU). Brewer and Vinson (1971) tested a number of inhibitors of melanisation and found PTU effective in reducing the encapsulation response of the host-parasite system studied. Nappi (1973) found that PTU incorporated into the diet of larval Drosophila olgonquin prevented melanisation of the hymenopterous parasite Pseudeucoila bochei parasite in the larvae.

A promising basis for future studies is the use of melanin precursors labelled with $[H_3]$ and $[C_{14}]$ at different positions of the molecule. Hempel (1966) used this technique to investigate the structure of melanin in malignant melanomas by employing DOPA- $[H_3]$ and DOPA- $[C_{14}]$.

This study has adopted a broad approach to the study of the host reaction of Anopheles labranchiae atroparvus elicited by Brugia patei. A variety of tritium labelled metabolites were employed to see whether any of them became incorporated into the pigment. The histochemical properties of the encapsulation material around the worms was also investigated.

Attempts were made to block melanin deposition around Brugia patei by injecting or feeding the host with inhibitors of melanisation/sclerotisation.

2. Cells possibly associated with melanogenesis

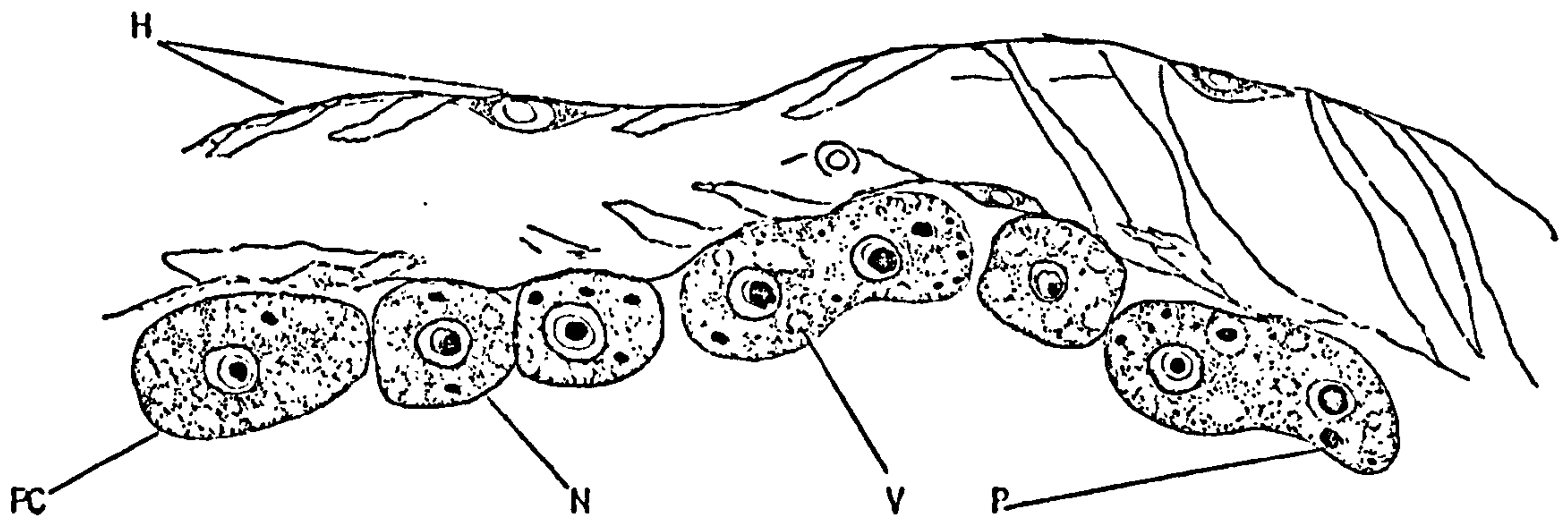
(a) Haemocytes

In many insects, cells involved in the genesis of melanin and the process of sclerotisation are the haemocytes (see Nappi, 1975). In the Diptera, blood cells appear to be few (Jones, 1962). Gotz and Vey (1974) found that aquatic dipterous larvae were able to encapsulate fungal spores without the involvement of haemocytes. Similarly the encapsulation of entomophilic, including filarial nematodes appears to take place in mosquitoes without any apparent haemocyte involvement, (Bronskill, 1962; Esslinger, 1962; Schacher, 1962a; Beckett, 1971; Poinar and Leutenegger, 1971; Laurence, 1970; Oothuman, Simpson and Laurence, 1974). Although the literature on the haemocytes of insects is quite extensive (Jones, 1962; Wittig, 1962; Arnold, 1974), apparently little attention has been given to the haemocytes of mosquitoes (Amouriq, 1960; Christophers, 1960). In this study, blood films from mosquitoes were examined with a view to being able to recognise types of blood cell, and to see whether any of the latter could be recognised in sections. It is possible that the reported absence of any observed involvement of blood cells in the defensive reaction of mosquitoes to filarial infection stems partly from difficulties of actually recognising a blood cell in section. The terminology of Jones (1962) was employed for identifying cells.

(b) Pericardial cells

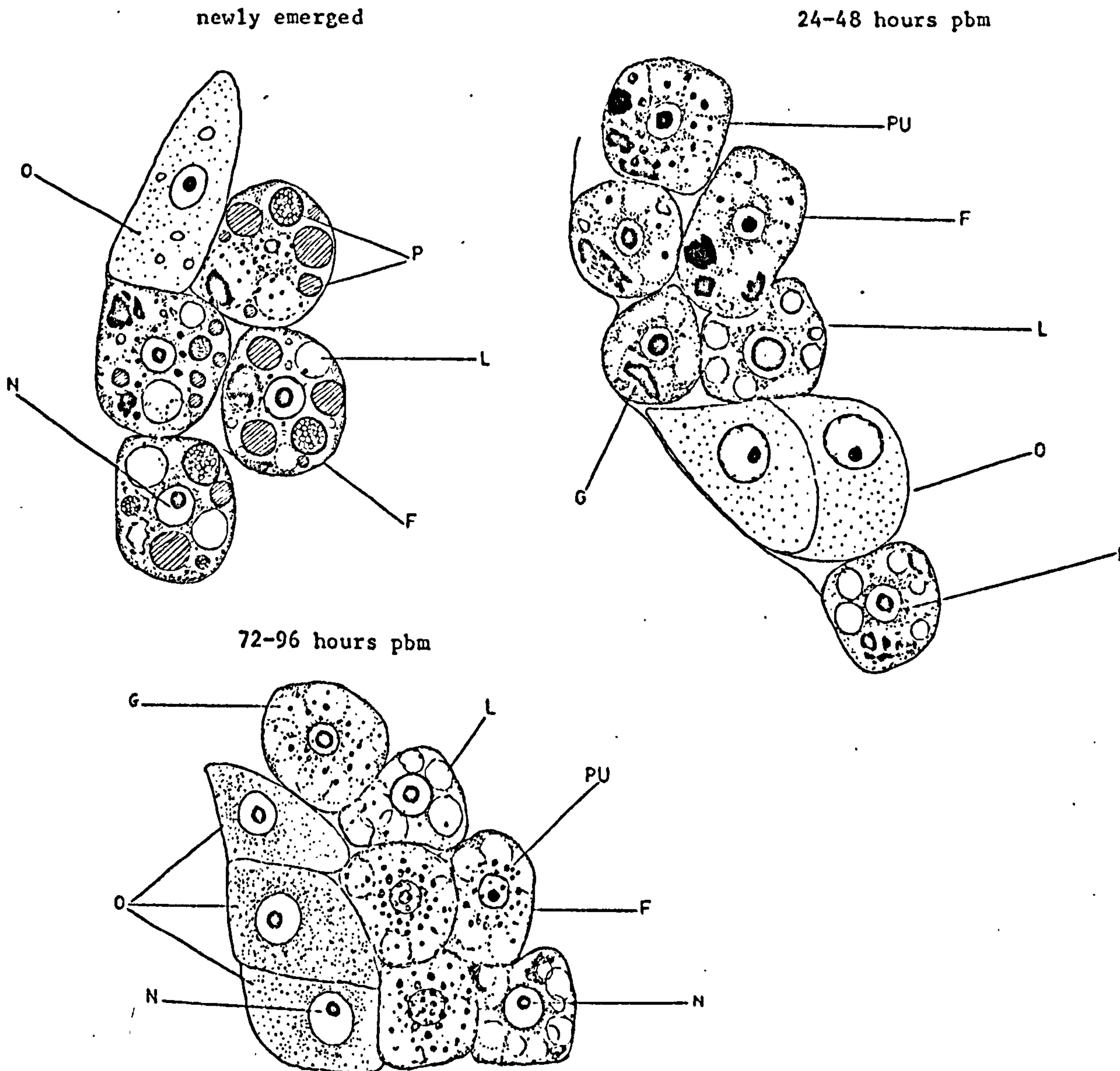
These cells form a linearly arranged discontinuous chain of multinucleate cells lying on either side of the heart (Fig 1a). Interest in them in relation to melanogenesis was stimulated by the observation that the highly vacuolated cytoplasm of these cells, often contained various inclusions, including a brown pigment, that was possibly melanin. In mosquitoes the histology of the pericardial cells of the Anopheles larva has been described by Imms (1908); that of the adult by Jones

Fig 1(a) Pericardial cells and heart of *Anopheles labranchiae atroparvus*: newly emerged female



PC = pericardial cell, H = heart, n = nucleus, v = cytoplasmic vacuole, p = pigment

(b) Fat body and oenocytes of *Anopheles labranchiae atroparvus*



f = fat body cell, o = oenocytes, n = nucleus, p = protein sphere, g = glycogen, l = lipid vacuole, pu = purine body

(1954; see also Christophers, 1960). The histology and function of the pericardial cells in various insects has been reviewed by Hollande (1922); their role in excretion by Maddrell (1971) and Cochran (1975). Pericardial cells are able to sequester materials such as foreign particulate matter, colloids and possibly amino acids from the blood, possibly returning some material back to the blood in a modified form (Wigglesworth, 1970a). Electron microscope studies on the pericardial cells have revealed the presence of structural modifications which enable these cells to fulfil such a role (Bowers, 1964; Hoffman, 1966; Sanger and McCann, 1968; Crossley, 1972). The paper by Crossley (1972) is especially valuable as this author has reviewed the more modern literature in some depth. These studies have tended to enhance the concept of the pericardial cells as possessing characteristics in common with the reticuloendothelial cells of vertebrates (Wigglesworth, 1970a).

(c) Oenocytes

These cells have also been considered in relation to melaninogenesis. Gnatzy (1970) has described the ultrastructure of the oenocytes of larvae of the mosquito Culex pipiens in relation to moulting. In general the oenocytes of insects have been shown to have ultrastructural characteristics which parallel the fine structure of vertebrate steroid producing cells (see Romer, 1974). Oenocytes in the mosquito are cells which comprise two populations, one fairly large, the other small (Fig 1b) situated in the ventro-lateral region of the abdomen. They are arranged in clusters, and in Anopheles there is a cluster either side of the first seven abdominal segments (Imms, 1908; see also Christophers, 1960). The oenocytes lie in the fat body and it would be unrealistic to consider them without reference to this important centre of intermediary metabolism.

(d) Fat body

There is considerable information on the changes in morphology of the insect fat body in response to growth and metamorphosis (Wigglesworth, 1972; Dutkowski, 1974), to ovarian maturation (de Loofe and Lagasse, 1970; Thomsen and Thomsen, 1974), on its role in lipid metabolism including storage (Zebe and McShun, 1959; Tietz, 1969) but of significant interest is the fact that the fat body serves as a site for the synthesis

and storage of glycogen and the metabolism of proteins (Wigglesworth, 1942; Kilby, 1963). Protein and nucleic acid metabolism in the fat body has been reviewed by Price (1973). In fact the pronounced function-related morphological changes that occur in the insect fat body, including that of mosquitoes (Fig 1b), appears to have been neglected by those workers who have pronounced upon fat body damage induced by filarial larvae (Lavôipierre, 1958; Esslinger, 1962; Schacher and Khâlil, 1968; Ho and Kan, 1972).

Recent work on the fat body of adult Aedes aegypti has shown that this organ synthesises yolk protein (vitellogenin) destined to be taken up by the developing oocyte (Hagedorn and Judson, 1972). The synthesis of vitellogenin is triggered by the blood meal, and RNA synthesis, rising as early as 2 hours after the blood meal, reached a peak by 28 hours before entering a sharp decline (Hagedorn, Fallon and Laufer, 1973). The mosquito fat body is thus proved to be a site for those biochemical mechanisms responsible for the elaboration of specific proteins, exportable into the haemolymph. This specific protein synthesis is under hormonal control (Fallon et al, 1974).

To summarise, the haemocytes, pericardial cells, oenocytes and fat body have been examined with the following limited objectives :

- (i) to record whether there are any aggregations of cells identifiable as blood cells near or adjacent to Brugia patei undergoing melanisation,
- (ii) to record the uptake of labelled metabolites with principle reference to labelled phenolic amino acids, into the haemocytes, pericardial cells, oenocytes and fat body.
- (iii) to collect histochemical data on these tissues and finally,
- (iv) to assess the significance of the autoradiographic and histochemical data in relation to melanogenesis of the host reaction.

RESULTS

In order to determine whether a particular radioactive compound became incorporated into the melanin pigment deposited around the parasite, each autoradiograph was thoroughly examined to note the presence of obviously marked pigment. In most cases labelled melanin plaques were too small in area to permit a proper quantitative assessment of the degree of incorporation. Some plaques were so intensely labelled that it would not have been possible to record the number of grains, even if the size of the plaques were large enough to allow this. In cases where it was not possible to conclude subjectively whether any incorporation had taken place into the pigment, the micrometer eyepiece with squares of 4 μm was employed. Grain counts were also made, within the same section of obviously unlabelled plaques. These counts were then compared with background counts collected for the section.

The incidence of melanisation in Brugia pateri-infected Anopheles labranchiae atroparvus is not high. In saline dissections the proportion of melanised larvae found is quite variable. In one experiment involving a sample size of 43 mosquitoes, 871 larvae were recorded, of which 158 were melanised. This is an incidence of just over 18%. Such a low figure has to be kept in mind when considering the autoradiographic data.

1. Incorporation of carbohydrate into the host encapsulation substance

There was no incorporation of glucosamine- $[\text{H}_3]$ into the pigment deposited around any of the worms present in the single female of Anopheles labranchiae atroparvus injected at 95 hours pbm. With glucose- $[\text{H}_3]$ and galactose- $[\text{H}_3]$, labelled melanin was observed over a few worms sampled from a total of 40 which has been grain counted. With glucose- $[\text{H}_3]$, 3 females were injected at each time interval of 24, 48, 72 and 96 hours pbm. Labelled pigment was observed in only 3 worms out of 31 sampled. A labelled worm was found at each time interval except 48 hours. In the latter case only 5 worms were sampled. With galactose- $[\text{H}_3]$ only one or two mosquitoes were autoradiographed per time interval. In this case, out of 9 worms where the melanin deposited around them was grain counted, only 1 worm was clearly labelled.

It is concluded that despite the low incidence of melanisation, it has been possible to demonstrate the active incorporation of $[H_3]$ -labelled glucose and galactose into the pigment of the host reaction. The failure to label the pigment with glucosamine- $[H_3]$ needs verification.

2. Incorporation of amino acids into the host encapsulation substance

Of the 14 amino acids used for injection into parasitised Anopheles labranchiae atroparvus (see Table 4b), only arginine- $[H_3]$, phenylalanine- $[H_3]$, tyrosine- $[H_3]$, tryptophan- $[H_3]$ and DOPA- $[H_3]$ became incorporated into melanin pigment deposited around the parasites. In the case of tryptophan- $[H_3]$ some doubt exists as to whether the labelled pigment observed was definitely associated with a parasite. There was no doubt whatsoever about the phenolic amino acids and arginine. These compounds had clearly become incorporated in plaques of melanin deposited around the parasites. Not all the melanin was labelled. There were worms surrounded by labelled pigment in close proximity to others which had pigment but no label at all. Yet when labelled melanin was present the plaques were often so intensely labelled that it was difficult, and sometimes impossible, to count the silver grains that were distributed over them. In a number of larvae, melanin deposition was localised at a single focus which was often the excretory pore whereas other larvae were almost entirely covered with a 'sheath' of pigment.

Each of the phenolic amino acids and arginine- $[H_3]$ were injected into parasitised Anopheles labranchiae atroparvus at 24, 48, 72 and 96 hours pbm, 3 animals being used at each time period, except for arginine- $[H_3]$ and phenylalanine- $[H_3]$ where only 1 or 2 animals were used. In this series, melanin plaques were seen which were large enough, yet not too dense to count (Plate 7). Without doubt, the counts obtained following the injection of either DOPA- $[H_3]$ or tyrosine- $[H_3]$ were greater than either of those obtained for phenylalanine- $[H_3]$ and arginine- $[H_3]$. For example phenylalanine- $[H_3]$ 24.0 ± 14.4 , arginine- $[H_3]$ 39.60 ± 3.84 , tyrosine- $[H_3]$ 93.20 ± 8.96 and DOPA- $[H_3]$ 94.80 ± 7.67 . Small eye piece graticule squares were used for these counts (1 unit area = $16 \mu m^2$) and at least 5 squares were counted for each isotope. Background counts per unit/area were less than 2 grains/square throughout.

In both host mosquitoes, radioactive phenols were incorporated into the integument, particularly around the bases of the wings. Marking was very intense with DOPA-[H₃]. The integument of uninfected as well as that of infected individuals was labelled. It was not possible to distinguish by grain counting, differences between exocuticle and endocuticle. All parts of the integument, including the hypodermis were apparently labelled.

3. Histochemical features of the host encapsulation substance

(a) Carbohydrate components

No staining of the encapsulation material with Schiff's reagent was observed unless the staining sequence was preceded by oxidation with periodate or sodium bismuthate. This indicated that staining was not due to native aldehydes present in the pigmented substance but was dependent upon reactive groupings engendered by the oxidation step. That the latter was due to periodate-engendered aldehydes was confirmed by the absence of staining in sections subjected to an aldehyde block, interposed between oxidation and the aldehyde detecting Schiff's reagent. To demonstrate that the positive PAS staining depended upon the presence of adjacent 1:2-glycol groups (-CHOH-CHOH) some sections were acetylated prior to carrying out the PAS technique. No PAS staining was observed in acetylated sections. If acetylated sections were saponified with ethanolic KOH before performing the PAS technique, then PAS reactivity was observed. This acetylation blockage of PAS reactivity followed by restoration resulting from saponification is a standard histochemical technique for demonstrating with near certainty, the carbohydrate nature of the test material (McManus and Mowry, 1960; Barka and Anderson, 1963; Lillie, 1965; Pearse, 1968; Chayen, Bitensky and Butcher, 1973). This carbohydrate-containing component of the host encapsulation substance did not appear to be glycogen. PAS staining persisted despite treatment of the sections with amylase prior to performing the technique. Not all the pigment was stainable after periodate oxidation and treatment with aldehyde detecting reagents.

No staining of the encapsulation substance was observed with techniques designed to demonstrate the presence of carboxylated or sulphated

Table 12 Histochemical properties of the encapsulation substance
deposited around Brugia pateri in Anopheles labranchiae
atroparvus

Carbohydrate components	Results
(a) 1:2 glycol rich substances	
Sodium bismuthate-Schiff	+
Sodium bismuthate-Schiff with amylase	+
PAS	+
PAS with amylase	+
Schiff's reagent	-
Acetylation-PAS	-
Acetylation-saponification-PAS	+
Periodate-aldehyde block-Schiff's reagent	-
(b) Polyanionic substances	
AB (2.5)	-
AB (1.0)	-
AB (CEC) 0.4-1.0M.	-
LID	-
HID	-
Protein components	
Bromphenol blue for basic proteins	-
DMAB-nitrite for tryptophan	-
Peracetic acid-Alcian blue for -SS groups	-
Mercury orange for -SH groups	-
Proteins rich in side chain carboxyls	-
Lipid components	
Oil red O for neutral fat	±
Pigment components	
Perl's prussian blue for iron	-
Massons fontana for melanins	+
Bleaching tests	
concentrated H ₂ SO ₄ (2 days)	Resistant
concentrated HCL (2 hours)	Resistant
40% peracetic acid (18-24 hours)	Resistant

Positive staining = +
 Negative staining = -
 Equivocal result = ±

polyanions. Polyelectrolytes appeared to be absent. This indicated that unless these groups were present but masked by association with basic protein (Quintarelli, 1963) it is safe to conclude that the 1:2 glycol-rich component of the encapsulation substance is a neutral mucosubstance (G mucosubstance, see Pearse, 1968).

(b) Protein components

The encapsulation substance varied in its reactivity towards protein-detecting histochemical techniques. The material was commonly reactive towards the dye solochrome cyanine but not towards bromphenol blue. Surprisingly, tests for indole groups were equivocal. The material did not stain with the DMAB-nitrite method for tryptophan, but its reactivity towards the diazotisation coupling method for tyrosine was variable. The staining reaction for tyrosine, using the Millon reaction, was exceptionally weak. No sulphhydryl or disulphide groups were demonstrated in the substance. Proteins rich in side chain carboxyl groups appeared to be absent.

It was concluded that the encapsulation substance contained protein material, the reactivity of which presumably depended upon the state of conjugation of the proteins with melanin pigment. With regard to the presumptive identification of the latter, the pigment was resistant to bleaching by a number of oxidising agents, and it was insoluble in the common laboratory solvents, such as xylene, chloroform, ethanol etc. The material reacted strongly towards the Massons fontana reaction, having a marked ability to reduce ammoniacal silver to the extent that the larvae were surrounded by intense black material.

(c) Lipid components

Oil red O staining was carried out on only a few individuals, as serial cryostat sections, although not difficult to produce, were time consuming. At the end of a session, it was a common experience to have many oil red O sections of parasitised mosquitoes but no melanised parasites. When the latter were present it was difficult to decide when a red smudge of stained material around a parasite represented a sudanophilic component of the encapsulation material, or was an artifact arising from fat debris.

The occurrence of sudanophilic material in the encapsulation substance needs further examination probably by the use of whole worms immersed in Oil red O. The present findings were inconclusive. The histochemical data is summarised in Table 12.

4. The effects of chemical inhibitors on melanisation around the worms

The dopa decarboxylase inhibitor (DL)-3-(3,-4-dihydroxyphenol)-2-hydrazino-2-methyl proprionic acid (α MDH) was injected into female Anopheles labranchiae atroparvus 24 hours after they had been fed on a cat infected with Brugia patei. Each mosquito was injected with less than 1 μ l of a solution of α MDH in Hayes saline. The compound was dissolved in the saline at concentrations of 0.5 and 1.0 μ g/ μ l. Controls were given saline alone. Mosquitoes were dissected at intervals following the injection and the numbers of melanised larvae assessed. The results are displayed in Table 13. Unfortunately in these inhibitor experiments the incidence of melanisation among control and treated larvae was small, and so subtle differences between the control and treated larvae would not be detected. The data obtained failed to indicate any significant difference between control and treated animals.

Unlabelled DOPA was injected into female Anopheles labranchiae atroparvus at the same concentration as that of the labelled solution to see whether the amino acid altered the level of melanisation of Brugia patei. It was considered to be a possibility since melanisation was found at 24 hours pbm the time of DOPA-[H₃] injection and earlier observations had shown that the worms did not become melanised until 48 hours pbm.

The analysis of the results from this experiment suggested that the addition of an extra melanin intermediate such as DOPA significantly increased the numbers of worms melanised compared to controls (Table 13).

β -alanine is involved in cuticle hardening and darkening (Neville, 1975). This amino acid was injected to see whether the incidence of melanised larvae was higher in the amino acid injected animals compared to controls. There was no evidence to suggest that this was so (Table 13).

Table 14 shows the effect of an oral dose of PTU and α MDH on the degree of melanisation of Brugia patei. With the dosing regime used there was

Table 13 The effect of injected α MDH, DOPA and B-alanine on the degree of melanisation of *Brugia patei* in *Anopheles labranchiae atroparvus*

CONTROL										α MDH (0.5 μ g/ μ l)			
Dissected p _{bm}	No mosquitoes used	No larvae found	No melanised	% melanised	No mosquitoes used	No larvae found	No melanised	% melanised	x ² square				
4	1	3	0		2	3	1						
5	1	2	1		2	6	1						
8	1	1	1		3	5	1						
9	1	2	1		1	2	0						
10	1	1	0		5	19	7						
Total	5	9	3	33	13	35	10	29	0.02				
α MDH (1.0 μ g/ μ l)													
4	1	2	1		3	6	5						
8	1	2	1		2	7	6						
9	1	4	2		3	7	3						
Total	3	8	4	50	8	20	14	70	0.32				
DOPA (0.8 μ g/ μ l)													
1	5	225	29		6	36							
2	3	88	18		2	25							
Total	8	313	47	15	9	61	25	41	20.51				
B-alanine (0.8 μ g/ μ l)													
					3	84	23						
					1	33	4						
					2	109	19						
					6	226	46	20	2.26				

NB Melanised = obviously melanised larvae including microfilariae visible in a saline dissection under the low power of the microscope.

no significant effect on melanisation around the larvae in the treated animals.

5. Histological and autoradiographical observations on haemocytes

Smears were taken from uninfected Aedes togoi and Anopheles labranchiae atroparvus and from individuals of these species infected with Brugia patei. Smears were taken during the period 1-6 hours and at 24-48 hours following the entry of the parasite into the host. The earlier time would cover the time that the microfilariae would be migrating through the haemocoel to reach the thoracic musculature. The later time covers the period during which melanisation is very much in evidence.

When the smears were examined care was exercised in obtaining a clear distinction between blood cells and the products of lysed or damaged cells that resulted from the method used to extract the blood. Fat body cells and their isolated nuclei were easy to recognise. Distinguishing between small fat cells and lipid containing blood cells (adipohaemocytes) was not possible. Jones (1975) states that adipohaemocytes are at least 10 times smaller than typical fat cells. This criterion does not apply to the mosquitoes studied here. In paraffin sections mosquito fat body cells show marked changes in morphology and size depending upon the physiological state of the individual.

Epithelial cells from damaged pieces of gut and salivary glands were recognisable as contaminants in smears. Purine granules released by damaged fat cells were distributed widely over the smears. With so much extraneous debris present, it was quite impossible to gain any information on differences that might exist between haemocytes from infected and uninfected mosquitoes. Changes in the haemocyte pattern which follow a parasitic infection is an area of much topical interest (see Nappi, 1975).

Despite the disadvantages inherent in using the glass capillary pipette to withdraw blood from adult mosquitoes, some data was obtained.

In both hosts, few blood cells were seen in smears taken from animals killed between 1-6 hours pbm. Haemocytes were more in evidence in smears prepared from animals killed at 24-48 hours pbm. In control animals smears

Table 14 The effect of an oral administration of the melanin inhibitor PTU, and α MDH on the degree of melanisation of *Brugia pateri* in *Anopheles labranchiae atroparvus*

CONTROL					PTU (1 µg/100 µls)				
Dissected pbm	No mosquitoes used	No larvae found	No melanised	% melanised	No mosquitoes used	No larvae found	No melanised	% melanised	x ² square
2	2	40	9		2	14	5		
3	4	63	14		7	115	24		
4	3	91	14		2	12	6		
7	4	44	12		4	64	8		
9	1	10	4		1	15	7		
Total	14	248	53	21	16	220	50	25	0.06

PTU (10 µg/100 µls)								
2	1	6	0	1	29	12		
3	1	33	19	2	64	15		
4	3	217	9	3	215	20		
7	2	19	11	3	67	28		
Total	7	275	39	9	375	75	20	3.32

α MDH (100 µg/100 µls)								
4	2	6	2	3	3	2		
7	4	12	10	5	14	14		
Total	6	18	12	8	17	16	94	2.58

NB Melanised = obviously melanised larvae including microfilariae visible in a saline dissection under the low power of the microscope.

The compounds were added to the sugar pads, with one compound being used per batch of mosquitoes.

made from animals killed at 24-48 hours after the blood meal contained few blood cells. Smears made from controls killed at 1-6 hours pbm were of poor quality. Of the types of cell recognised, these were assigned to one of four types :

- (i) Minute cells, less than 3 μm . These cells contained a rounded nucleus surrounded by a thin rim of cytoplasm. In other insects, larger but similar cells are classified as prohaemocytes.
- (ii) Pleomorphic cells of various sizes, with rounded nuclei. These cells were observed readily in smears made from the blood of Anopheles labranchiae atroparvus, and were present in uninfected as well as infected animals. These cells commonly were fusiform in shape and are regarded here as being plasmatocytes.
- (iii) Cells with eccentrically placed nuclei, and often with a pyriform shape were regarded as being oenocytoids.
- (iv) Small cells with a highly vacuolated cytoplasm were regarded as probably being adipohaemocytes.

In conclusion, blood cells were recognised in these smears despite the presence of so much cellular debris. The presence of cell types in some smears but not others is probably an artifact, but it did appear that the most prominent cell type were fusiform plasmatocytes in Anopheles.

Turning now to an examination of blood cells in sectioned material. Blood cells were observed in sections taken from Anopheles labranchiae atroparvus. The animals had been injected with 1% trypan blue and killed at 30-60 minutes later. The sections were dewaxed and mounted in balsam without staining. Haemocytes were seen associated with the heart and along the edge of organs, lying close to the basement membranes. These cells were rounded and not fusiform. In size these cells lie within the range 2-3 μm in diameter to larger cells up to 12 x 10 μm in size. In sections stained with CHP it was not possible to pick out blood cells with certainty. Small cells assumed to be blood cells were often seen lying close to or adhering to the basement membranes which

invest the internal organs. No cells were observed in the heart.

In sections of infected Anopheles labranchiae atroparvus no clearly recognisable blood cells were seen in association with filarial larvae present within the thoracic flight muscles. This was so even in animals which had been injected with 1% trypan blue. In the course of this and related studies, hundreds of serially sectioned, infected mosquitoes of both host species have been available for study. The unequivocal presence of haemocytes in close association with developing filarial larvae was not seen.

It is clear that the probability of distinguishing haemocytes in routine sections is slight. However, labelled blood cells were observed in some of the autoradiographs prepared from mosquitoes injected with DOPA-[H₃]. Hardly any DOPA-[H₃] labelled haemocytes were seen in autoradiographs of Aedes togoi, but in Anopheles labranchiae atroparvus that had been injected at either 24, 48, 72 or 96 hours pbm the label over the haemocytes was very intense. The cells were a dense black colour (Plates 6 and 8a). Three animals were injected at each time interval, and labelled cells were seen at all time intervals and in all 12 animals. A good saggital section was sampled from one animal per time interval and all the labelled blood cells present were counted.

There were fewer labelled blood cells present in the head than in the thorax and abdomen. More than 65% of the haemocytes were present lying adjacent to the basement membrane of an organ. The sections sampled from the 24 hour old animal had fewer marked cells in total than those present in sections from animals killed at 48-96 hours pbm. At 24 hours there were more marked cells counted in the thorax as a percentage of the total number of cells present in the section, it was found that the percentage of DOPA-[H₃] marked cells in the thorax increased throughout the period 48-96 hours pbm. At 24 hours 38% of the cells were present in the thorax, 41% at 48 hours, 51% at 72 hours and 69% at 96 hours pbm. Correspondingly, the percentage of DOPA-[H₃] marked cells present in the abdomen steadily decreased throughout the period under study.

The principal tissues associated with these cells were the thoracic flight muscles, pericardial cells and fat body. At 24 hours the cells counted

were mainly associated with the pericardial cells and heart. At the later stages the cells were predominantly associated with the thoracic fat body and flight muscle. In one of the animals injected at 24 hours, DOPA-[H₃] marked blood cells were observed lying quite close to a worm but out of the 12 mosquitoes used in this experiment not one single worm was found which showed DOPA-[H₃] marked blood cells adjacent to a worm.

One of the principal features noted in this series was the presence of brown material, presumably melanin, present in the cytoplasm of a number of tissues. Some of the cells adhering to, or an integral part of, the small tracheae were also intensely labelled with DOPA-[H₃], tyrosine-[H₃], phenylalanine-[H₃] and thymidine-[H₃]. These cells often had a fusiform shape, similar to those cells classified as plasmatocytes present in the blood smears. In unlabelled cells of this type, the cytoplasm was vacuolated.

In the experiment carried out to record the distribution of DOPA-[H₃] in uninfected Anopheles labranchiae atroparvus the general level of label was higher than in the infected controls. These animals were killed at 93 hours pbm. Surprisingly, there was little sign of DOPA-[H₃] labelled blood cells either in infected or control animals. The few that were observed in the infected animals were associated with the heart.

It is concluded that the marking of blood cells with DOPA-[H₃] is a variable phenomenon in the refractory mosquito. In infected individuals where marked cells are abundant, there is a strong association with the thorax, which in the sections analysed indicated that the level of cells in the thorax rises during the period 1-4 days pbm.

6. Incorporation of radioactive precursors into the pericardial cells, heart, oenocytes and fat body

(a) Uptake of nucleosides

Thymidine-[H₃] marking of the pericardial cells, heart, oenocytes and fat body was absent or slight in Aedes togoi and Anopheles labranchiae atroparvus during the early period of infection by Brugia patei.

Furthermore, individual variation in marking was recorded between individuals of the same stage pbm. In some individuals a proportion of the fat body cell population was intensely marked. Some oenocytes had very heavily labelled nuclei, more so than that of the surrounding fat cells, but most of the oenocytes were unlabelled.

Following the injection of uridine-[H₃] into Aedes togoi parasitised with Brugia patei label over the pericardial cells, heart, oenocytes and fat body was low in animals that had been injected at either 26.5 or 45 hours pbm. In mosquitoes injected at 5, 6 or 8 days pbm label over the oenocytes and heart remained low, although the label over the pericardial cells and fat body was much higher compared with the earlier times. The mean grain counts over the fat body are depicted in Table 15. As only one or two animals were autoradiographed at each time interval, the data has to be treated with a degree of caution. Yet it is plausible that the observed increase in uridine-[H₃] incorporation by the fat body at 5, 6 and 8 days pbm reflects an elevation in nucleic acid synthesis.

A crude estimate of the relative proportion of fat body cells involved in the uptake of uridine-[H₃] at these different time intervals was made by counting the proportion of labelled fat body cells (5+ grains, with the counting unit square centred over the fat cell nucleus) observed within a randomly chosen microscope field. From such estimates it appeared that in the individuals sampled at 26.5 hours and 45 hours pbm, only 40-60% of the fat body cells showed incorporation of uridine-[H₃]. The fat body cells sampled at 5.6 and 8 days were 100% labelled.

(b) Uptake of carbohydrate

The uptake of glucose-[H₃], galactose-[H₃] and glucosamine-[H₃] was low in the pericardial cells, heart, oenocytes and fat body of both host mosquitoes. Of these 4 cell types, the label over the pericardial cells was higher. The degree of label was uniform in that there was no observed change in uptake of glucose-[H₃] and galactose-[H₃] label in Anopheles labranchiae atroparvus when autoradiographs from animals injected at 24, 48, 72 or 96 hours pbm were compared.

Table 15 Incorporation of uridine-[H₃] into the fat body
of Aedes togoi and Anopheles labranchiae atroparvus

Time injected (pbm)	Mean grain density \bar{x} ± standard error of mean	
	Fat body	Background
(a) <u>Aedes togoi</u>		
26.5 hours	4.20±1.30	2.03±0.10
45 hours	10.20±0.91	1.79±0.11
5 days	20.00±1.67	1.90±0.10
6 days	29.80±3.89	1.96±0.15
8 days	31.40±2.73	1.97±0.15
(b) <u>Anopheles labranchiae atroparvus</u>		
45 hours	9.20±1.30	4.70±0.20

One mosquito was sampled per time sequence.

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x})
per unit area of fat body = $\frac{(\text{total number grains counted})}{5 \text{ unit areas}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{(\text{total number grains counted})}{100 \text{ unit areas}}$

1 unit area = 64 μm^2 .

(c) Uptake of amino acids

The relative degree of incorporation of $[H_3]$ -labelled amino acids into the pericardial cells (Plates 8b, 10), oenocytes (Plate 9) and fat body (Plate 10) of both host mosquitoes is set out in Table 16. As only one mosquito of each species was autoradiographed for each amino acid, differences between individuals must be ignored. The purpose here was to record which of the 4 cell types within an individual appeared to be most active in terms of amino acid uptake. In general the oenocytes exhibited a more intense label of amino acid (Plate 9). The fat body cells were the next most consistently heavily labelled cells (Plate 10). This pattern of incorporation was similar in both mosquito hosts. In Aedes togoi that had been injected with leucine- $[H_3]$ and pulsed at varying time intervals, good labelling of the fat body and oenocytes was observed in animals that had been given a pulse as short as 2 minutes.

In the refractory mosquito, phenylalanine- $[H_3]$ was used as a marker to determine whether there was any pronounced difference in incorporation of labelled amino acid among the 4 cell types, when sampled from uninfected animals. The results are set out in Table 17 and suggest that uptake of phenylalanine- $[H_3]$ into the fat body of infected individuals is slightly higher than that of the ^{un}infected controls.

Table 18 also records the mean grain counts obtained for each of the 4 cell types when the refractory and infected mosquito was injected with either tyrosine- $[H_3]$ or DOPA- $[H_3]$. The uptake of phenolic label by the pericardial cells apparently bore no relationship to the time of injection. Incorporation was uniform throughout. With phenylalanine- $[H_3]$ and tyrosine- $[H_3]$ the label over the heart was more intense during the period 24-48 hours pbm compared with the means obtained at 72-96 hours pbm. With DOPA- $[H_3]$ the situation was reversed. The mean grain density over the heart was low at 24-48 hours but higher at 72-96 hours pbm.

The oenocytes of both infected and uninfected Anopheles labranchiae atroparvus readily incorporated phenylalanine- $[H_3]$ at 24, 48, 72 and 96 hours pbm. In both infected and uninfected individuals, phenylalanine- $[H_3]$ label was high at 96 hours pbm compared to neighbouring fat body cells (Table 17). The oenocytes of infected Anopheles labranchiae atroparvus

Table 16 Incorporation of [H_3]-amino acid into pericardial cells, oenocytes and fat body of (a) *Aedes togoi* (b) *Anopheles labranchiae atroparvus*: when parasitised by *Brugia patei*

Isotope	Time of injection (hours pbm)	Mean grain density $\bar{x} \pm$ standard error of mean			
		Pericardial cells	Oenocytes	Fat body	Background
(a) <u>Aedes togoi</u>					
Histidine	96	-	-	6.20 \pm 1.33	2.35 \pm 0.13
Proline	120	3.80 \pm 0.33	-	1.80 \pm 0.37	0.83 \pm 0.09
Arginine	92	-	66.40 \pm 5.63	14.60 \pm 1.53	<3
Lysine	92	11.40 \pm 1.60	22.0 \pm 1.91	23.80 \pm 5.50	<1
Methionine	95	12.0 \pm 0.74	44.0 \pm 1.62	5.0 \pm 1.01	1.99 \pm 0.14
Cystine	99	-	25.20 \pm 2.99	22.0 \pm 2.38	5.21 \pm 0.23
Iso-leucine	91	86.0 \pm 6.40	-	\times 100	<1
Leucine	93	-	-	53.80 \pm 1.72	2.71 0.31
Valine	90.5	-	52.0 \pm 3.16	26.60 \pm 1.99	<1
Phenylalanine	92	6.40 \pm 1.89	13.0 \pm 2.32	9.80 \pm 1.20	3.02 \pm 0.15
Tyrosine	117	-	15.0 \pm 1.22	9.80 \pm 1.45	2.83 \pm 0.74
Tryptophan	91.5	19.0 \pm 1.80	41.0 \pm 5.01	33.40 \pm 1.31	0.64 \pm 0.07
Hydroxytryptophan	98	-	10.02 \pm 0.74	4.80 \pm 1.24	4.42 \pm 0.18
(b) <u>Anopheles labranchiae atroparvus</u>					
Histidine	96	4.80 \pm 0.65	7.0 \pm 0.74	6.20 \pm 0.80	2.76 \pm 0.12
Proline	120	6.60 \pm 1.0	8.60 \pm 1.51	4.0 \pm 0.74	2.02 \pm 0.13
Arginine	92	-	35.60 \pm 2.97	18.20 \pm 2.21	2.56 \pm 0.13
Lysine	92	>100	>100	>100	<1
Methionine	95	30.40 \pm 1.40	69.40 \pm 4.9	13.40 \pm 1.66	<1
Cystine	99	-	-	3.80 \pm 1.03	3.80 \pm 0.25
Iso-leucine	91	54.40 \pm 3.60	>100	>100	<1
Leucine	93	29.60 \pm 4.28	91.0 \pm 2.57	38.60 \pm 3.65	2.27 \pm 0.13
Valine	90.5	57.20 \pm 1.58	-	66.60 \pm 2.70	<1
Phenylalanine	92	37.30 \pm 4.20	>100	63.20 \pm 4.18	3.44 \pm 1.19
Tyrosine	117	-	16.80 \pm 1.03	11.20 \pm 1.65	2.23 \pm 0.11
Tryptophan	91.3	11.20 \pm 1.53	27.2 \pm 1.53	28.0 \pm 2.57	<1
Hydroxytryptophan	98	-	14.80 \pm 1.45	6.90 \pm 1.41	4.41 \pm 0.15

One mosquito of each species was used per time interval.

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x})
per unit area of muscle = $\frac{(\text{total number grains counted})}{5 \text{ unit areas}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{(\text{total number grains counted})}{100 \text{ unit areas}}$

1 unit area = 64 μm^2 .

- = no observations. In such cases the pericardial cells were too fragmented to count, and the oenocytes contained refractile granules which made it too difficult to count the silver grains.

incorporated tyrosine-[H₃] at 24, 48 and 72 hours pbm, but incorporation of DOPA-[H₃] was slight (Table 18).

The tyrosine-[H₃] label over the fat body was interesting. Grain counts taken from 96 hours injected animals also showed a further decline in label. The results are set out in Fig 2. Thus whereas in both infected and uninfected Anopheles labranchiae atroparvus the mean number of grains over the fat body of phenylalanine-[H₃] injected animals did not appear to vary at the different times sampled, the ability of the fat body to incorporate tyrosine-[H₃] steadily decreased within the time range studied. No similar decrease was recorded for the oenocytes. There was little incorporation of DOPA-[H₃] by either the oenocytes or fat body of parasitised Anopheles labranchiae atroparvus within the time period studied.

To summarise these findings it was found that labelling with phenolic amino acids of the pericardial cells of Anopheles labranchiae atroparvus was not easily related to the time of injection. Phenylalanine-[H₃] and tyrosine-[H₃] uptake into the heart of this mosquito was higher at 24-48 hours compared with that observed at 72-96 hours. Conversely, DOPA-[H₃] incorporation by the heart was lower at 24-96 hours compared to that observed at 72-96 hours. At 96 hours pbm there was a difference between uptake of labelled amino acids by the oenocytes compared with the fat body cells. The label over the oenocytes was generally higher. In the fat body, phenylalanine-[H₃] incorporation appeared to be constant with time and was slightly higher in infected animals. In Anopheles labranchiae atroparvus, tyrosine-[H₃] uptake declines steadily within the time period 24-96 hours pbm.

7. Histochemical observations on pericardial cells, heart, oenocytes and fat body

A histochemical analysis of the pericardial cells, oenocytes and fat body produced sharp distinctions between these different cell types. The heart tissue was omitted as it is predominantly composed of muscle fibres (see Jones, 1954; Christophers, 1960) and there were no special features of histochemical significance observed for this tissue in contrast to the other cell types mentioned.

Table 17 Incorporation of phenylalanine-[H₃] into pericardial cells, heart, oenocytes and fat body of *Anopheles labranchiae atroparvus* (a) parasitised by *Brugia patei* (b) uninfected controls

Time of injection (hours pbm)	Mean grain density $\bar{x} \pm$ the standard error of mean				
	Pericardial cells	Heart	Oenocytes	Fat body	Background
(a)					
24	47.60±3.83	64.80±2.42	44.80±3.29	76.40±5.20	<2
48	38.40±7.80	80.60±6.79	-	62.20±4.59	<2
72	45.20±5.25	39.20±4.18	<100	74.60±3.30	3.11±0.19
96	34.40±3.61	18.80±1.11	87.60±2.47	41.20±7.26	<2
(b)					
24	35.20±1.48	58.80±5.38	55.20±2.80	41.80±2.95	<2
48	12.20±3.31	83.60±6.08	24.40±1.53	35.0 ±3.79	<2
72	45.40±4.25	15.0 ±1.57	48.80±4.02	34.80±2.52	<2
96	33.80±2.55	25.0 ±2.56	<100	33.80±3.65	<2

Each insect was pulsed for 30 minutes before being killed.

One uninfected and one infected insect was used at each time interval.

Mean grain density (\bar{x})
per unit area of tissue = $\frac{\text{(total number grains counted)}}{5 \text{ unit areas}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{\text{(total number grains counted)}}{100 \text{ unit areas}}$

1 unit area = 64 μm^2 .

- = no observations.

(a) Pericardial cells

With Kurnick's methyl green pyronin technique, pyronin staining of the cytoplasm of the pericardial cells of Aedes togoi and Anopheles labranchiae atroparvus was weak throughout the period 1-7 days pbm. There was no detectable difference in pyronin staining between infected and uninfected females. The cytoplasmic pyronin reactivity was removed by a pretreatment with ribonuclease indicating that the dye was binding to RNA.

Malonin's sodium bisulphite toluidine blue and the PAS technique indicated that glycogen was present in the cytoplasm of the pericardial cells of newly emerged females of Aedes togoi but not in newly emerged females of Anopheles labranchiae atroparvus. The pericardial cells of newly emerged females of Aedes togoi also contained a PAS positive 1:2 glycol-rich substance that, unlike glycogen, was not removed by pretreatment with amylase. A material with similar staining properties was not observed in newly emerged females of Anopheles labranchiae atroparvus. In blood-fed females of both species, including individuals parasitised with Brugia pateri, the pericardial cells were consistently unreactive to PAS or other glycol-detecting methods. No polyanionic material was similarly demonstrated in infected and uninfected individuals of both these species. With the AB(CEC) technique, slides of both species immersed in dye baths containing salt concentrations of up to 0.8M MgCl stained the cell nuclei of the mosquitoes, including those present within the pericardial cells, a bright blue. The cytoplasm was stained very weakly by comparison. This type of background staining was usually encountered when using the CEC method and is non-specific.

The pericardial cells were often so heavily vacuolated, especially in Aedes togoi, that the cell cytoplasm was reduced to a thin rim around the nuclei. Despite this it was apparent that these cells contained no acid mucosubstance.

Granules which stained positively for protein were occasionally present in the pericardial cells of females of Anopheles labranchiae atroparvus, but usually no stainable inclusions were present. The commonest inclusions observed in the pericardial cells of both species, including infected individuals, were brown granules. The latter were enclosed in

Table 18 Incorporation of tyrosine-[H₃] and DOPA-[H₃] into pericardial cells, heart, oenocytes and fat body of Anopheles labranchiae atroparvus

Isotope	Time of injection (hours pbm)	Mean grain density $\bar{x} \pm$ standard error of mean				
		Pericardial cells	Heart	Oenocytes	Fat body	Background
Tyrosine	24	35.10±4.44	77.00±14.30	45.38±3.48	62.20±2.91	<2
	48	17.10±1.85	60.20± 1.11	26.10±2.62	44.33±4.85	<2
	72	20.10±1.82	14.00± 1.85	37.80±2.67	25.60±1.08	2.83±0.87
DOPA	24	61.80±7.54	5.80± 1.39	7.60±1.32	2.40±0.49	<2
	48	57.33±6.82	8.20± 1.14	4.00±0.71	3.47±0.43	<2
	72	71.40±5.73	18.87± 2.61	3.70±0.88	3.53±0.53	<2
	96	53.50±3.99	17.30± 1.75	2.60±1.04	4.73±0.52	<2

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x})
per unit area of tissue = $\frac{\text{(total number grains counted)}}{\text{number mosquitoes sampled} \times 5 \text{ unit areas}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{\text{(total number grains counted)}}{\text{number mosquitoes sampled} \times 100 \text{ unit areas}}$

1 unit area = 64 μm^2 .

vacuoles and of variable size. These pigment-containing inclusions were concentrated mostly in the cortical regions of the pericardial cells. The pigment was unreactive to Perl's prussian blue method but was coloured an intense black with the Masson Fontana method. This pigment presumably represents globules of melanin. In a number of the autoradiographs prepared from animals of both species that had been injected with phenolic amino acids, brown pigment was present in a variety of cell types, not just the pericardial cells.

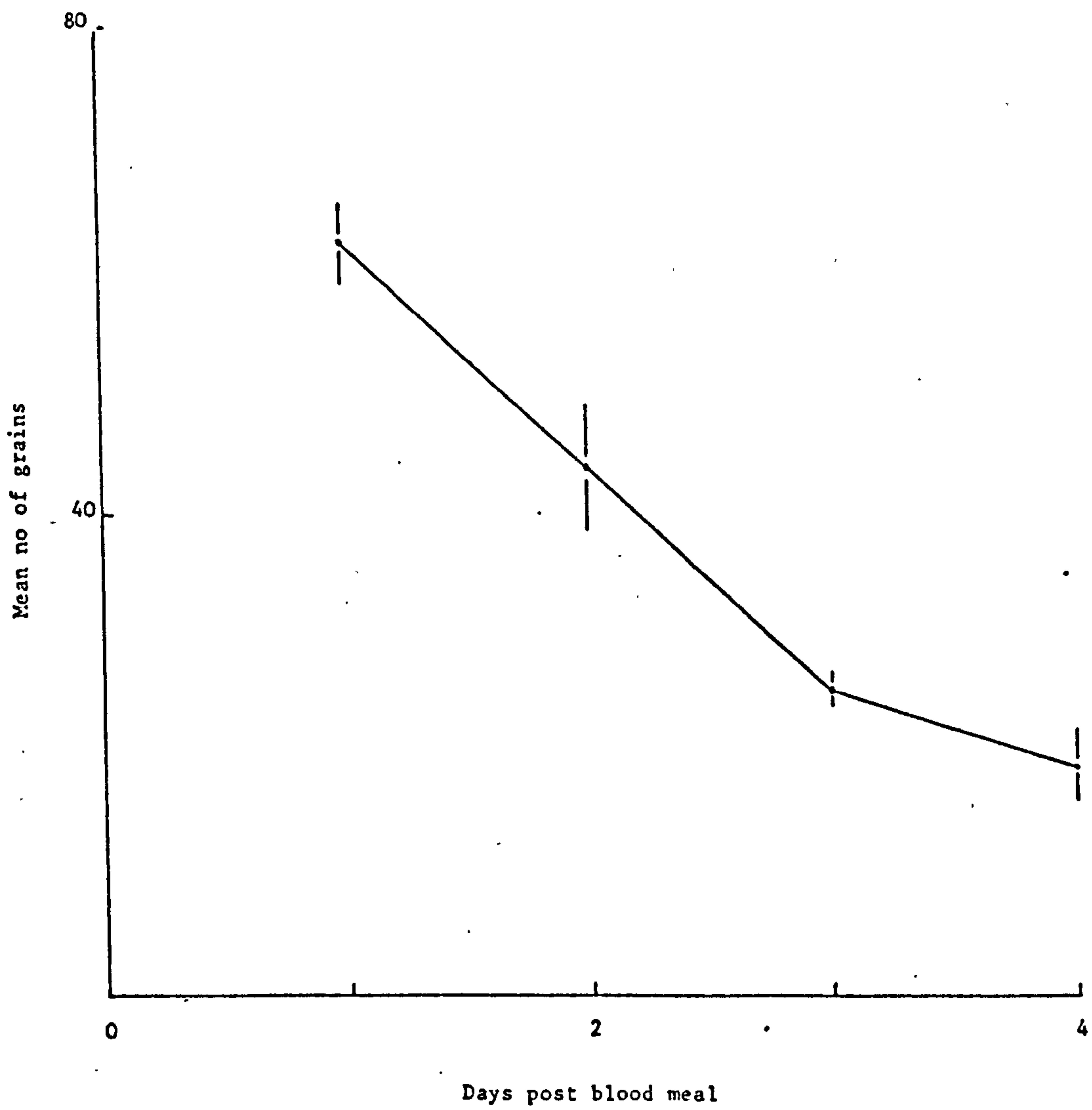
In female Anopheles labranchiae atroparvus that had been injected with a solution of 1% trypan blue the pericardial cells appeared to have largely disintegrated. In those instances where such disintegration had not occurred the pericardial cells were packed with the dye.

In cryostat sections, the pericardial cells were not particularly easy to detect. There is less shrinkage of the tissues in a cryostat section and in the blood-fed specimens used, the expanded abdominal midgut considerably reduced the haemocoel volume. The pericardial cells were pressed close to sheets of parietal fat body. Even so, no neutral fat, non-specific esterase and glucosaminidase was demonstrated in such specimens. In conclusion it is noted that the general lack of histochemical reactivity of the pericardial cells was not altered by the presence of the filarial parasite.

(b) Oenocytes

In newly emerged Anopheles labranchiae atroparvus and Aedes togoi, the oenocytes tended to be elongated in shape, but in older blood-fed individuals the shape was more pyriform, sometimes rounded. The following observations apply to both large and small types of oenocytes present in infected and uninfected females. The oenocytes stained positively with pyronin in blood-fed females. The staining was absent in sections that had been pretreated in ribonuclease, indicating that the pyronin reactivity was due to RNA. At 24-48 hours pbm, pyronin staining of the cytoplasm was weak (Plate 11a), but at 3-7 days pbm, the staining was stronger (Plate 11b), indicating that the RNA content of the oenocytes had risen.

Fig 2 Incorporation of tyrosine-[H₃] into fat body of *Anopheles labranchiae atroparvus*



Each point represents the mean of 2-3 mosquitoes.
Each bar represents the standard error of the mean.

No 1:2 glycol rich material, polyanionic material and the enzymes, non-specific esterase and β -glucosaminidase were demonstrated in the oenocytes. Intense Oil red O staining of the surrounding fat body tended to obscure the sudanophilic properties that the oenocytes may have had. Small globules of fat appeared to be present in the cytoplasm of the oenocytes of some individuals, but these could have been derived from lysis of adjacent fat cells caused by the sectioning artifact. In FA-fixed sections the oenocytes were often found to contain small vacuoles arranged around the rim of the nucleus. The cytoplasm of the oenocytes stained strongly with copper pthalocyanine, indicating the presence of phospholipids.

The most significant histochemical feature of the oenocytes that was consistently recorded was the intense protein staining of these cells, particularly with techniques used to demonstrate basic proteins such as Solochrome cyanine and mercuric bromphenol blue. The cytoplasm was stained a deep uniform colour with such techniques. In CHP sections the oenocytes were intensely acidophilic, staining deeply with phloxine. This intense avidity for protein stains was undiminished in infected individuals. Oenocytes were reactive to protein stains in newly emerged individuals and in those that had developed and laid eggs.

Initially, an attempt was made to determine whether the numbers of oenocytes visible in sections rose during the period of infection and whether there was any difference in the visible numbers of oenocytes in sections of uninfected mosquitoes, compared to those infected with Brugia patee. The mean number of oenocytes visible/per section was calculated for both uninfected and control individuals. At least 5 sections were sampled from each mosquito and several mosquitoes were used at each time interval (24 and 48 hours pbm). With this crude system of measurement no significant difference in the number of oenocytes observed, was found between infected and control mosquitoes in either of the two species.

(c) Fat body

The following observations apply equally to infected and uninfected female Aedes togoi and Anopheles labranchiae atroparvus. Pyronin staining of the nucleoli and cytoplasm of the fat body cells (trophocytes) was intense at 24-48 hours pbm in comparison to the staining at 3-7 days pbm

where pyronin staining in the fat cells was negligible (Plate 11). Since the pyronin reactivity of the fat body cells was absent in sections pretreated with ribonuclease, the changing intensity of pyronin staining was regarded as reflecting a pattern of RNA changes in the fat body.

In general the cytoplasm of the fat body cells contained material which reacted intensely with Best's carmine, sodium bisulphite-toluidine blue, sodium bismuthate-Schiff and the PAS technique (Plate 12). With these techniques the reactivity of the fat cells was prevented by prior incubation of the sections in amylase, indicating that the material in the cells was glycogen. The presence of this storage polysaccharide in the fat body varied between individuals, but in general was present in individuals from emergence and throughout egg development. Glycogen was also present in the fat body of individuals that were infected with Brugia patei. However, in some individuals that contained either mature filarial larvae of Brugia patei, or abnormal larvae of this worm alive at 10-14 days pbm, the fat body was depleted of glycogen. Apart from glycogen, no other neutral carbohydrate was detected in the fat body, and acid mucosubstances were absent in blood fed individuals.

Oil red O staining of cryostat sections of Aedes togoi and Anopheles labranchiae atroparvus indicated that the cytoplasm of the fat body cells was rich in globules of neutral fat. The latter was commonly demonstrable in individuals at emergence and to at least a week after blood feeding.

Protein containing spheres were present in the fat body of newly emerged individuals but were absent from the fat body of those coming to feed on blood. During the course of the blood meal the fat body did not incorporate protein storage spheres. The fat body contained little acid phosphatase and no β -glucosaminidase activity, but was rich in non-specific esterase. The fat body of Anopheles labranchiae atroparvus reacted strongly to Masson's Fontana technique, the cytoplasm containing masses of black material indicating that substances capable of reducing the nitrate to silver, such as melanins were present.

To summarise the major histochemical findings (Table 19), it was found that the RNA staining patterns of the oenocytes and fat body suggested that the RNA content of the oenocytes increased, whereas that of the fat body declined

Table 19 Histochemistry of Pericardial cells, oenocytes and fat body of *Anopheles labranchiae atroparvus*

Technique	Newly emerged (<24 hours)			24-48 hours pbm			72-96 hours pbm		
	Pericardial cells	Oenocytes	Fat body	Pericardial cells	Oenocytes	Fat body	Pericardial cells	Oenocytes	Fat body
<u>Nucleic acids</u>									
Methyl green pyronin	+	+-	++	+	-	+++	+	++	+
Ribonuclease control	-	-	-	-	-	-	-	-	-
<u>Carbohydrates</u>									
Best's carmine	+-	+-	+++	-	-	+++	-	-	+++
Amylase control	-	-	-	-	-	-	-	-	-
Sodium bisulphite toluidine blue	+-	+-	+++	+	-	+++	-	-	+++
Amylase control	-	-	-	-	-	-	-	-	-
PAS	-	-	+++	-	-	+++	-	-	+++
Amylase control	-	-	-	-	-	-	-	-	-
Acetylation-PAS	-	-	-	-	-	-	-	-	-
Acetylation-saponification + PAS	-	-	-	-	-	++	-	-	++
PAS with aldehyde blockade	-	-	-	-	-	-	-	-	-
AB(2.5)	-	-	-	-	-	-	-	-	-
AB(1.0)	-	-	-	-	-	-	-	-	-
LID	-	-	-	-	-	-	-	-	-
HID	-	-	-	-	-	-	-	-	-
AB(CEC)*	-	-	-	-	-	-	-	-	-
0.2M	-	-	-	-	-	-	-	-	-
0.6M	-	-	-	-	-	-	-	-	-
1.0M	-	-	-	-	-	-	-	-	-
<u>Proteins</u>									
Bromphenol blue	+	+++	+++	+	+++	-	+	+++	-
DMAB-nitrite	+	+	+	+	+	+	+	+	+
Peracetic acid-AB	+	+	+	+	+	-	+	+	-
Mercury-orange	+-	+-	+-	+-	+-	+-	+-	+-	+-
Diazo-coupling	+-	+-	+++	+-	+-	-	+-	+-	-
Millons	-	-	-	-	-	-	-	-	-
Mixed anhydride	-	-	-	-	-	-	-	-	-
Massons fontana				++	-	+++	++	-	+++
<u>Lipids</u>									
Oil red O	+-	+-	+++	+-	+-	+++	-	+-	+++
Copper phthalocyanin**	+	+++	+	+	+++	+	+	+++	+
<u>Enzymes</u>									
Acid phosphatase	+	+	+	+	+	+	+	+	+
β -glucosaminidase				-	-	-	-	-	-
Non-specific esterase				-	-	+++	-	-	+++

Intense staining = +++, moderate staining = ++, positive staining = +, equivocal staining = +-, negative staining = -. A blank space in the table indicates that no observations of the tissue were made at that particular time interval.

* background staining only.

** probably background, non specific staining.

after the blood meal. The oenocytes were rich only in basic protein whereas the fat body of newly emerged and blood fed females contained substantial depots of glycogen and neutral fat. Only the fat body of newly emerged individuals contained reserves of protein. The pericardial cells had little histochemical reactivity.

DISCUSSION

The host reaction of Anopheles labranchiae atroparvus is detectable as plaques of brown pigment deposited on the surface of Brugia patei. It may be an over simplification to consider that this substance consists solely of melanin pigment. The autoradiographic data has established that the host reaction, ie the encapsulation substance is derived, at least in part, from phenolic amino acids and the basic amino acid arginine. The phenolic amino acids, phenylalanine, tyrosine and DOPA are known intermediates of melanin and cuticular proteins (Thomsen, 1962; Hempel, 1966; Hackman, 1974).

In addition to melanin pigment the encapsulation substance contains a carbohydrate component rich in 1:2 glycol groups, and glucose-[H₃] and galactose-[H₃] are probably incorporated into this component rather than into melanin.

Histochemically, no evidence was found to suggest the presence of polyanionic material in the encapsulation substance, but the presence of acid mucosubstance marked by association with basic protein (Quintarelli, 1963) cannot be excluded.

In insects, acid mucosubstances have not been studied in detail (Baldwin and Salthouse, 1959; Kato and Sirlin, 1963; Ashhurst and Richards, 1964; Joshi, 1965; Marshall, 1966; 1968; Mustafa and Kamat, 1972; Ashhurst and Costin, 1971; Sharief, Perdue and Dobrogosz, 1973) and there is no evidence which links acid mucosubstances with an involvement in the immune response in insects. In comparison, acid mucosubstances feature in the reaction complex which arises in molluscs parasitised by nematodes (Cheng, 1975).

In insects, neutral mucosubstances appear to have a role to play in the production of the immune response. In Periplaneta americana, parasitised by acanthocephalan larvae, Robinson and Strickland (1969) recorded the presence of 1:2 glycol-rich material in the reaction complex around the parasite. Brennan and Cheng (1975), in a study on the resistance of Moniliiformis dubius to the defence reactions of Periplaneta americana, associated the presence of divalent cations on the glycocalyx of the

parasite with its successful inhibition of the tyrosinase activity of the host. In insects parasitised by parasitoids the ionic nature of the surface of the parasitoid is believed to play a role in the encapsulation process (Salt, 1970; Brewer and Vinson, 1971; Vinson, 1974).

The surface of the microfilaria of Brugia patei contains polyanions and 1:2 glycol groups (Simpson and Laurence, 1972), and some attention should be given towards determining whether the surface properties of this parasite represent a significant factor in the evasion or suppression of the host reaction of the mosquito.

The presence of 1:2 glycol-rich material in the encapsulation substance of Anopheles labranchiae atroparvus is interesting in that it differs from that produced by other mosquito species. Schacher (1962b) obtained a negative PAS reaction in the encapsulation substance of Anopheles quadrimaculatus and Psorophora confinnis parasitised by Brugia pahangi. The encapsulation substance elicited by larvae of the midge Chironimus against fungal spores is also PAS unreactive (Gotz and Vey, 1974). According to Lillie (1965) melanins in mammals vary in their response to 1:2 glycol detecting techniques, but are usually PAS negative. In more primitive vertebrates, melanin-macrophages in Teleost fish are PAS positive, (Roberts, 1975).

Possibly the 1:2 glycol-rich component of the encapsulation substance of Anopheles labranchiae atroparvus represents a stage in the formation of melanin containing plaques. As carbohydrate is not involved in the indole melanin pathway (Thomsen, 1962) it is suggested that the 1:2 glycol-rich component represents the residues of a glycoprotein carrier used to transport melanin intermediates to the site where melanin synthesis is occurring. In Periplaneta americana, diphenyl glucosides bind blood proteins destined for incorporation into sclerotizing cuticle (Koeppe and Mills, 1972; Koeppe, Mills and Brunet, 1974). In Aedes aegypti parasitised by the nematode Neoaplectana carpocapsae, a homogenous non-pigmented deposit always appeared on the surface of the parasite before any deposition of melanin (Poinar and Leutenegger, 1971).

In insects the physiological and chemical basis for melanisation and sclerotisation are poorly understood, and it is usually not possible to

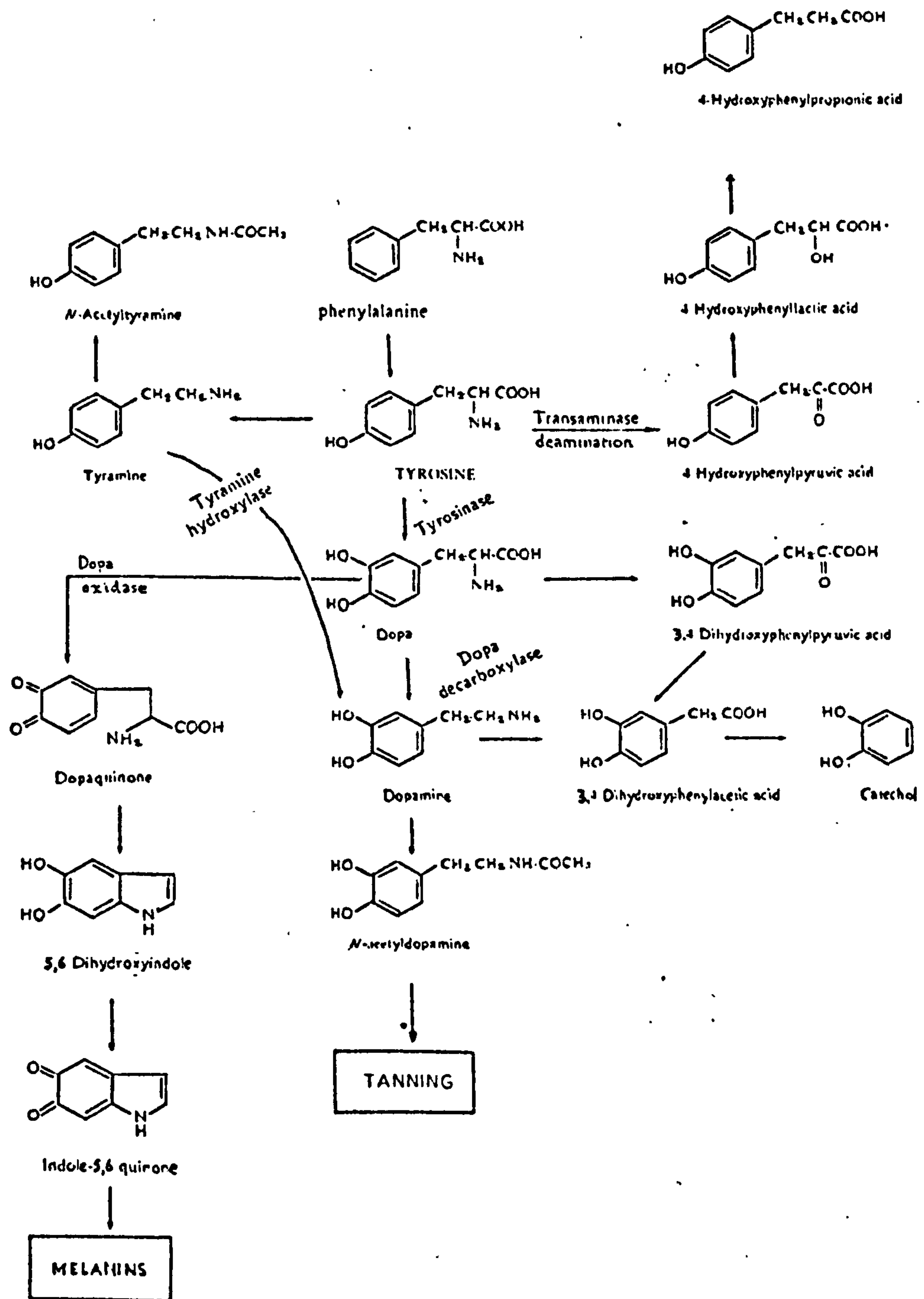
disentangle the process of melanisation occurring around a helminth parasite and damaged integument from events which form an integral part of the tanning sequence (Hackman, 1974). Melanisation and sclerotisation are processes which utilise phenolic intermediates, and both events may occur at the same tissue locus (Hackman, 1974). Differentiating between these two processes at the morphological level, is rarely possible, particularly as current histochemical techniques cannot make a distinction between the different phenoloxidasases which mediate these two processes (see Lipke, 1975; Neville, 1975).

A generalised scheme which indicates possible relationships between melanisation and tanning is set out in Fig 3. In this scheme phenylalanine is hydroxylated to tyrosine which in turn can be hydroxylated to DOPA (Brunet, 1967); Seligman and Doy, 1972). Tyrosine can also be converted to the -O-phosphate (Hodgetts and Konopka, 1973), to tyramine (Karlson and Hervlich, 1965) or to phenolic acids (Mills and Lake, 1971). DOPA can be oxidised by dopa oxidase as a preliminary to entering the indole-melanin pathway (Thomsen, 1962), or be converted to dopamine by dopa decarboxylase (Sekeris and Karlson, 1966). Dopamine undergoes acetylation to N-acetyldopamine which is a widespread if not universal tanning agent (Karlson and Sekeris, 1962; 1964), although other tanning agents are known (Brunet, 1967). N-acetyl-dopamine reacts with quinones before it is cross linked (tanned) to proteins (Hackman, 1974). How much of this general scheme is applicable to mosquitoes has yet to be determined.

It is clear from Part 4 that first stage larvae of Brugia patei cause little detectable flight muscle damage, even in refractory Anopheles labranchiae atroparvus, and yet melanisation of the larvae still ensues. The wound response data showed that phenolic amino acid incorporation and melanin formation are correlated with obviously damaged muscle, possibly with the release of an injury factor (see Lai Fook, 1966; Berry, et al, 1967; Cherbas, 1973) yet in Part 6 it is recorded that microfilariae of Brugia patei which migrate through the wall of the stomach produce lesions in the epithelial wall. This damage to the midgut wall is not followed by melanisation of the epithelial cells.

Obvious questions which require an explanation are why the parasite is

Fig 3 Schematic presentation of possible relationships between sclerotisation and melanisation in mosquitoes



melanised by the host even though the parasite causes no detectable damage which could stimulate the release of an injury factor, and why a detectable gut lesion is not followed by melanisation, whereas obviously damaged flight muscle is rapidly melanised.

It is suggested that in the unfavourable environment of the refractory host, substances released by the parasite serve to attract an excess of quinones to the area. It is known that the Drosophila mutant Ebony converts excess quinones into melanin by spontaneous polymerisation (Hodgkett and Konopka, 1973). The surface of the parasite could act as a template for condensation of quinones into melanin. Hydrolytic enzymes may feature in this postulated scheme. Melanisation frequently was present only at the excretory and anal vesicles. Terwedow and Huff (1976) recorded intense acid phosphatase activity in these regions from microfilariae of Wuchereria bancrofti. Melanisation of damaged flight muscle may be related to the fact that the hypodermis suffers damage during the process of thoracic injection. Pieces of cuticle and hypodermal cells are thrust deep into the wound. These damaged fragments of hypodermis could well act as primers for melanin synthesis, the trigger possibly being an injury factor released from the damaged cells. The insect hypodermis is closely involved in the synthesis and subsequent sclerotisation of new cuticle (Locke, 1974), and this process requires a supply of phenolic metabolites (Hackman, 1974; Weis-Fogh, 1970; Neville, 1975), Aedes togoi and Anopheles labranchiae atroparvus have been shown to incorporate phenylalanine-[H₃], tyrosine-[H₃] and DOPA-[H₃] into the integument. The resolution was insufficient to enable a distinction to be made between label appearing over the cuticle from that over the hypodermis. This phenolic uptake was independent of the presence of the parasite, but it is not known whether the incorporation was related to the fact that the integument had been damaged by the injection technique, or just represents little more than a turnover pattern of phenolic proteins. It is possible that the incorporation is necessary to meet the demands of normal wear and tear, particularly in areas of wing bases where resilin may be situated (see Kristenson, 1966).

With regard to the damaged midgut (Part 6) it is suggested that the reparative mechanisms do not entail the release of an injury factor, and so melanisation is not initiated. From the standpoint of the different

locations of the lesions, it is obviously advantageous for the insect to be able to seal off a wound at its body surface quite quickly, and also to introduce antibacterial agents into the wounded area. If successful, this act would prevent dessication and minimise damaging effects of any invading micro-organisms. Melanin is believed to have bacteriocidal properties (Edelstein, 1971). In the lower vertebrates immuno mechanisms are simpler than in mammals. For example, melanins are found in Teleost macrophages which are associated with long standing bacterial ulceration (Roberts, 1975).

Data in Part 4 has shown that melanisation of damaged muscle results well within 30 minutes of the initial insult, yet melanisation of Brugia patei in Anopheles labranchiae atroparvus is not evident until 18-48 hours pbm. It is tempting to relate this latent response with the relatively low state of activity of the parasite during this period of its development (Feng, 1936; Laurence and Simpson, 1971). The autoradiographic data on the parasite (Part 3), suggests, that compared with later times, incorporation by the 24 hour old larvae is low. Once the parasite undergoes active growth, substances may be released from it which actively promote the onset of a host response.

An obvious point to consider is that in the susceptible host Aedes togoi, Brugia patei can complete its development because it is able to completely suppress the onset of the mosquito's host response, whereas in a refractory host, such as Anopheles labranchiae atroparvus, the parasite is able to effect only a partial suppression. In the latter mosquito DOPA-[H₃] incorporation into all tissues examined was significantly higher in unparasitised individuals (see Parts 4 and 6). This finding would tend to support the hypothesis of some type of suppression mechanism. However, in Aedes togoi, DOPA-[H₃] incorporation was minimal, even in unparasitised individuals. Phenylalanine-[H₃] incorporation between unparasitised and Brugia patei infected Anopheles labranchiae atroparvus was similar, except in the fat body where incorporation was significantly higher in the infected animals.

Recent work by Andreadis, Flanagan and Kaczor (1975) does not support the hypothesis that melanisation can be suppressed by the parasite. Aedes aegypti is refractory to Reesimermis nielsenii, but susceptible to Neoaplectana

carpocapsae, Andreadis, Flanagan and Kaczor (1975) infected larvae Aedes aegypti first with Reesimermis nielsenii and then challenged the mosquitoes with an infection of Neoaplectana carpocapsae. The latter were melanised. This is evidence which shows that a mermithid nematode does not evade the host response by a general inhibition of melanisation. The possibility of a suppressive mechanism acting locally at the cuticular surface cannot be excluded.

If prevention of melanisation is effected locally at the surface of the nematode's cuticle, an inhibition of the action of enzymes involved in surface melanisation, would be an effective way of doing this. Preventing a build up of quinones at the worm's surface would be essential if spontaneous condensation into melanin is to be avoided.

Prime enzymes involved in melanisation/sclerotisation include dopa-decarboxylase which converts DOPA to dopamine (Sekeris and Karlson, 1966), β -glucosidase which releases N-acetyl-dopamine from its glucoside (Sekeris, 1964), Acetyl CoA transferase which converts dopamine to N-acetyldopamine (Karlson and Sekeris, 1964). Tyrosinase (dopa oxidase, catechol oxidase) is active against DOPA, dopamine and N-acetyldopamine (see Brunet, 1967; Neville, 1975). In the fleshfly, Sarcophaga, cyclic AMP was shown by Seligman and Doy (1972) to stimulate the in-vivo hydroxylation of tyrosine to DOPA.

A number of chemical inhibitors effective against melanisation/sclerotisation have been used (Brewer and Vinson, 1971; Hayes, Johnson and Schechter, 1975; Lipke, 1975; Nappi, 1975; Neville, 1975). Adding α MDH or PTU to the sugar pads or injecting α MDH into Anopheles labranchiae atroparvus, did not prevent the formation of melanin around Brugia patei, and at damaged muscle sites. Conversely, the egg shells of the α MDH injected mosquitoes failed to darken when the eggs were laid. Schlaeger and Fuchs (1974a) have shown that α MDH does block dopa-decarboxylase. These workers consider that darkening of the egg shell is due to sclerotisation not melanisation. This conclusion is open to question. However it is clear that in Anopheles labranchiae atroparvus, melanisation around Brugia patei does not depend upon dopa-decarboxylase activity.

The present study has not provided much insight into the location of a cellular template for melanin synthesis. A complicating factor is that any cell which is in direct contact with the haemolymph and which possesses a micropinocytotic capacity, could take up melanin sub-units, with the result that brown pigment could accumulate in the cell. The site where melanin synthesis was initiated might well go undetected. The presence of unlabelled pigment in many cells from phenolic amino acid-injected animals might be linked to this. Ultrastructural work on Aedes togoi and Anopheles labranchiae atroparvus might help to clarify sites where pinocytotic mechanisms are operable.

In many insects haemocytes have the capacity not only to phagocytose particular matter, but also are involved in the encapsulation and melanisation of metazoan parasites (see Salt, 1963, 1968, 1970).

In this study the histological data has clearly established the absence of any small cells associated with the development of the encapsulation material around Brugia patei parasitic in Anopheles labranchiae atroparvus. This absence of a recognisable cellular response fits the pattern described previously in an earlier communication (Oothuman, Simpson and Laurence, 1974) and by other workers engaged on histopathological studies of filarially infected mosquitoes (Esslinger, 1962; Schacher and Khalil, 1968; Townsen, 1970; Beckett, 1971; Ho and Kan, 1973).

The autoradiographic results do not indicate whether the small, melanotic, DOPA-[H₃]-labelled bodies that were associated with the wall of the heart, and with various basement membranes, represent attached, melanin-synthesising haemocytes or aggregates of protein phenols precipitated out of the blood by fixation. In Periplaneta americana, Koeppe and Mills (1972) demonstrated that protein bound phenols were translocated from the haemolymph into the integument during and after ecdysis. In mosquitoes, yolk protein (vitellogenin, see Part 7) is synthesised in the fat body (Hagedorn and Judson, 1972; Hagedorn, Fallon and Laufer, 1973) and transported into the haemolymph where it is taken up by pinocytosis into the oocyte of the current follicle (Anderson and Spielman, 1969). It is possible that a variety of acellular, protein specific carrier systems exist in mosquito haemolymph.

Apart from blood cells, a melanin synthesising capacity could be present in the pericardial cells, hypodermal cells, oenocytes, fat body and follicular epithelium. Cells were observed to contain brown granules, especially in animals that had been injected with phenolic amino acids. The possible function of the hypodermal cells in relation to the wound response was mentioned earlier. As filarial nematodes can develop in males of susceptible strains of mosquitoes but not in refractory males, (Terwedow and Rodriguez, 1973; Townson, 1974), it is unlikely that the follicular epithelial cells play any part in supplying phenolic metabolites for the encapsulation substance, although incorporating tyrosine-[H₃] and DOPA-[H₃] during vitelline membrane formation.

Although the pericardial cells incorporated uridine-[H₃], pyronin staining of these cells was low throughout the period of the filarial infection. Furthermore, despite the, at times, intense incorporation of DOPA-[H₃] the amino acid label over the pericardial cells was unrelated to the presence of the filarial larvae or to the time of the injection. In view of these findings it is considered unlikely that the pericardial cells contribute towards melanisation of filarial larvae. The uptake of labelled precursors probably reflects the scavenging ability of the pericardial cells to sequester material from the haemolymph, including surplus proteins (see Wigglesworth, 1970a).

In the oenocytes the uptake of uridine-[H₃] was low, but RNA staining increased after 48 hours pbm. In terms of amino acids and proteins there was a tendency for the amino acid label over the oenocytes to be greater than that over the fat body cells and the oenocytes consistently gave strong reactions for basic proteins and phospholipids. In other insects oenocytes synthesise structural lipids and may be involved in ecdysone synthesis (see Neville, 1975). If the oenocytes of mosquitoes have a similar function, then clearly they are unlikely to be directly involved in the production of melanotic proteins.

In the fat body there was a decrease in cytoplasmic RNA staining after 48 hours pbm whereas uridine-[H₃] incorporation increased. Recently, Hagedorn, Fallon and Laufer (1973) have shown that the level of total RNA in the fat body of female Aedes aegypti shown a three fold increase

shortly after the blood meal followed by a rapid decline. These workers postulated that the RNA synthesis is an obligatory pre-requisite for vitellogenin synthesis to take place. In addition to producing mRNA for the transcription of vitellogenic protein it is possible that the fat body elaborates RNA which is involved in the construction of melanin proteins unless the latter already exist in the fat body as storage forms. The fat body of blood-fed Anopheles labranchiae atroparvus reacts positively to Masson's fontana technique indicating that reducing substances such as melanin are present. These could conceivably be derived by micropinocytotic activity of the fat body cells^{as} mentioned earlier. Certainly, in blood-fed Aedes togoi and Anopheles labranchiae atroparvus, the fat body is depleted of stainable protein reserves. The latter are readily identified as small globules in the fat body of newly emerged females. On balance, the view here is that the mosquito synthesises melanin protein as and when required for the encapsulation of filarial larvae, and does not draw upon a 'melanin store'.

The fat body of Aedes togoi and Anopheles labranchiae atroparvus incorporated a variety of labelled amino acids, some of which are probably converted into carbohydrate (see Wigglesworth, 1942; Price, 1973). The fat body in newly emerged and blood fed individuals generally contains glycogen and neutral fat. The biochemically detectable increase in amounts of glycogen and triglycerides that occurs in mosquitoes following a blood meal (Nayer and Saurman, 1975b) takes place in the fat body and eggs (Part 7). The presence of non-specific esterase activity in the fat body is probably associated with lipid metabolism (see Geering, 1975).

With phenolic amino acids, the fat body incorporated minimal amounts of DOPA-[H₃] but did incorporate phenylalanine-[H₃] and tyrosine-[H₃]. With tyrosine-[H₃] the fat body of Anopheles labranchiae atroparvus showed a decreasing ability to incorporate the isotope within the period 24-96 hours pbm. Whether this pattern of tyrosine-[H₃] incorporation is related to the presence of Brugia patei, or is a function of ovarian development needs investigating further. There was a tendency for the fat body of uninfected Anopheles labranchiae atroparvus to incorporate lesser amounts of phenylalanine-[H₃] than fat body from parasitised individuals.

If the fat body does possess cells coded to produce quinone proteins that are transportable to focal points for melanin synthesis, that encapsulation does not normally occur before 24-48 hours pbm may be related to the prior commitment by the fat body to produce vitellogenin. The relationship of the fat body to melanin manufacture warrants further investigation, particularly as it will lend itself to study by in-vitro technique.

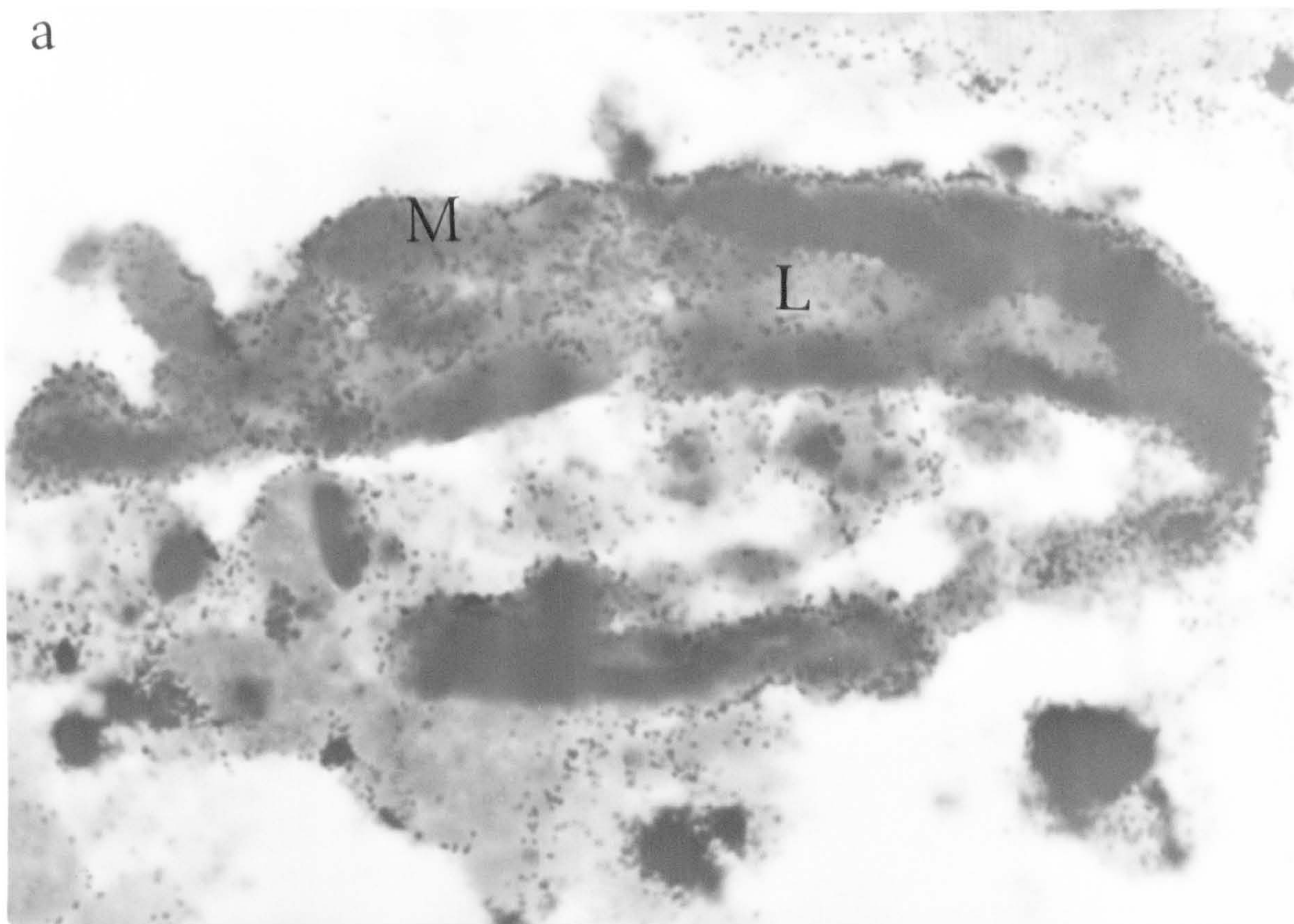
Along with the fat body, the tracheole cells also deserve further investigation. The tracheole cells readily incorporate thymidine-[H₃] and labelled phenolic amino acids. Brown pigment was present in many of these cells. Poinar and Leutenegger (1971) recorded that tracheole cells were frequently associated with melanin deposits around Neoplectana carpocapsae when parasitic in larval Culex pipiens. It is possible that the tracheole cells are attached haemocytes.

PLATE 7

Incorporation of DOPA-[H₃] into encapsulation substance surrounding Brugia patei in Anopheles labranchiae atroparvus. F = flight muscle. L = larva. M = labelled melanin pigments.

- (a) injected at 24 hours pbm.
- (b) injected at 93 hours pbm.

a



b

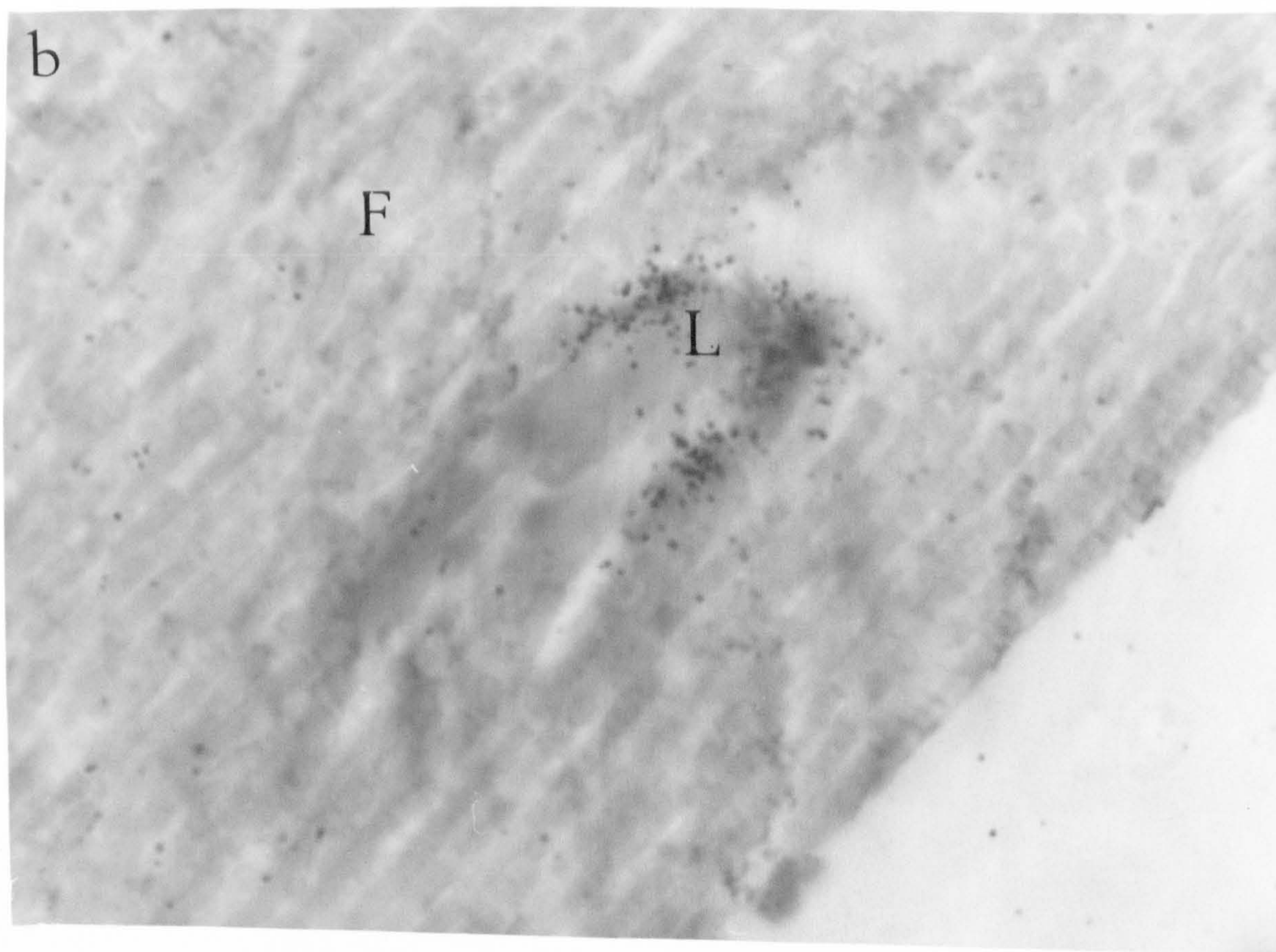


PLATE 8

Incorporation of DOPA-[H₃] into (a) haemocytes and (b) pericardial cells of Anopheles labranchiae atroparvus parasitised by Brugia patei. F = flight muscle.
h = haemocytes. PC = pericardial cell. m = melanin granule. FB = fat body cell. This individual was injected with the isotope at 72 hours pbm.

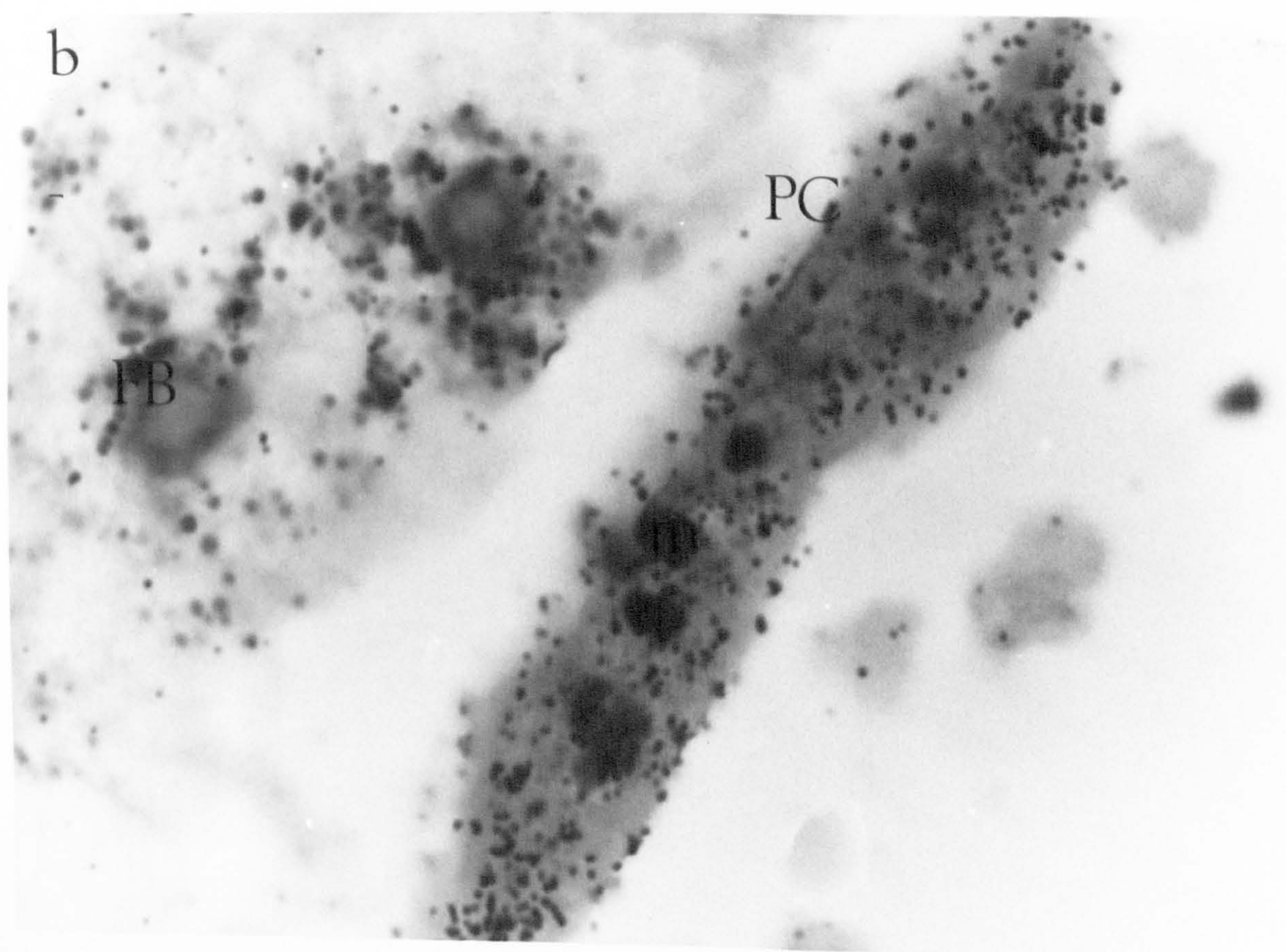
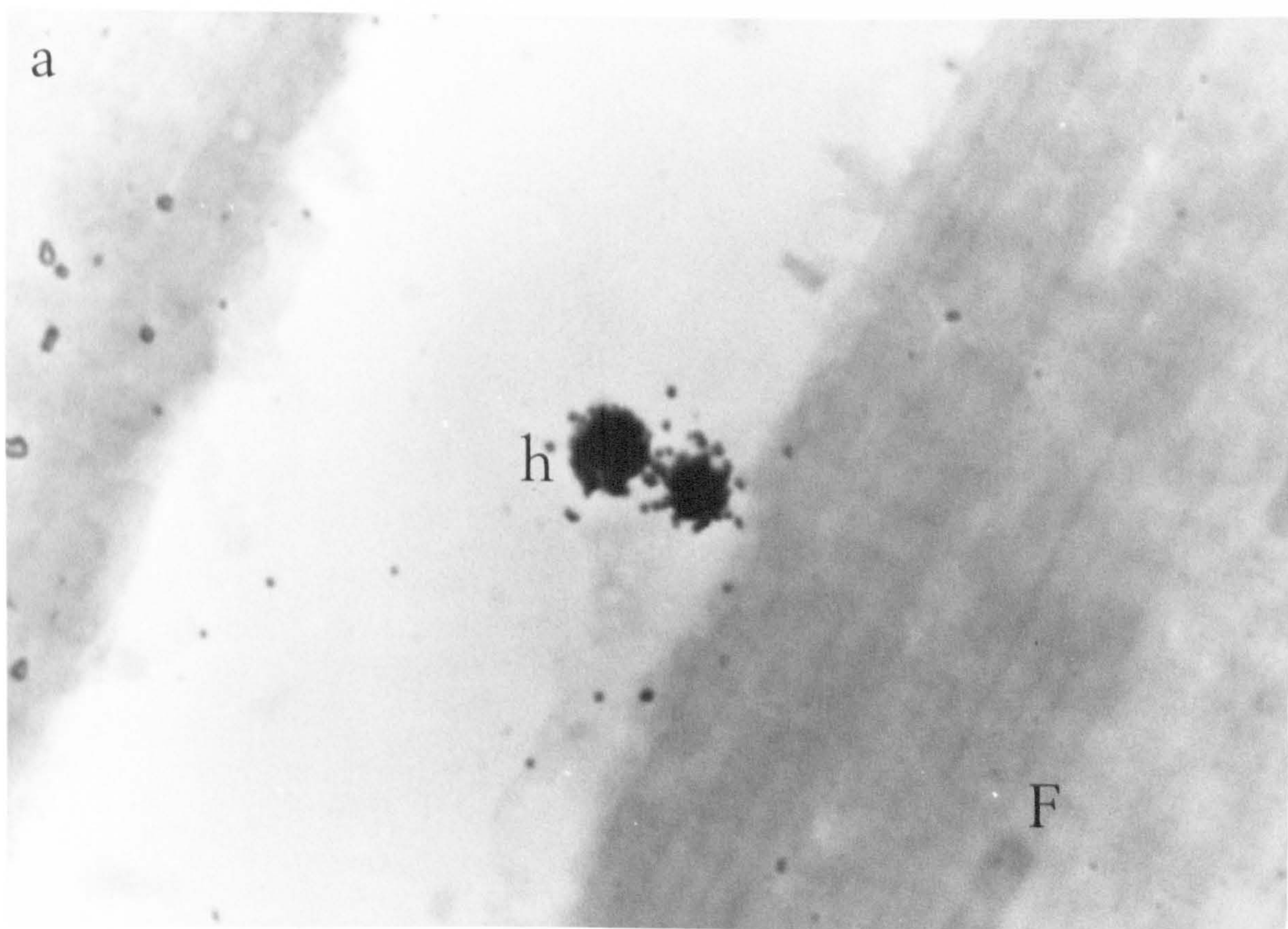


PLATE 9

(a) and (b). Incorporation of arginine-[H₃] into oenocytes of Anopheles labranchiae atroparvus. The isotope was injected at 96 hours pbm. oe = oenocyte. fb = fat body cell. In (a) the focus is on the silver grains, in (b) on the oenocyte and fat cells.

(c) Incorporation of lysine-[H₃] into oenocytes of Aedes togoi. The isotope was injected at 92 hours pbm.

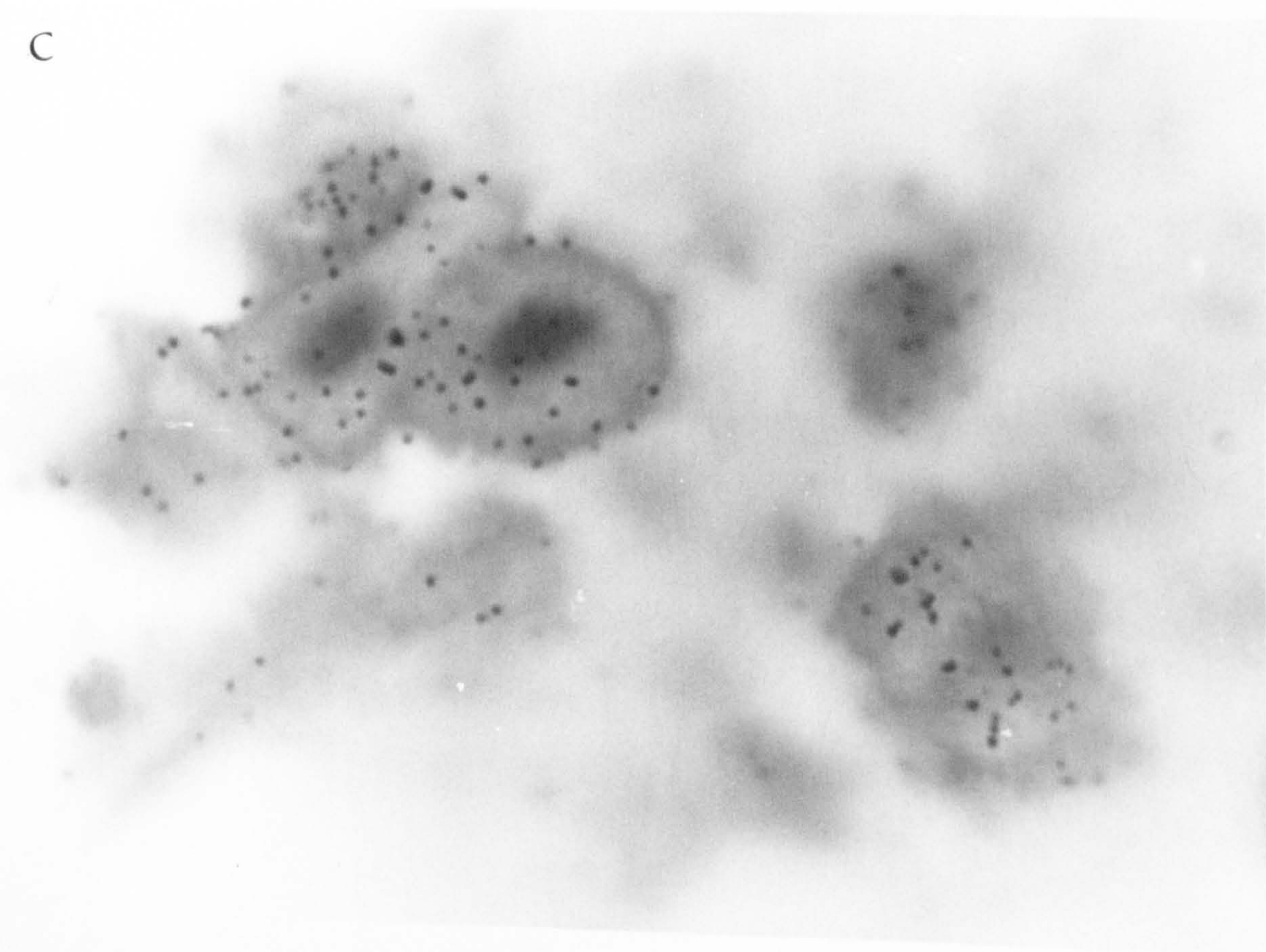
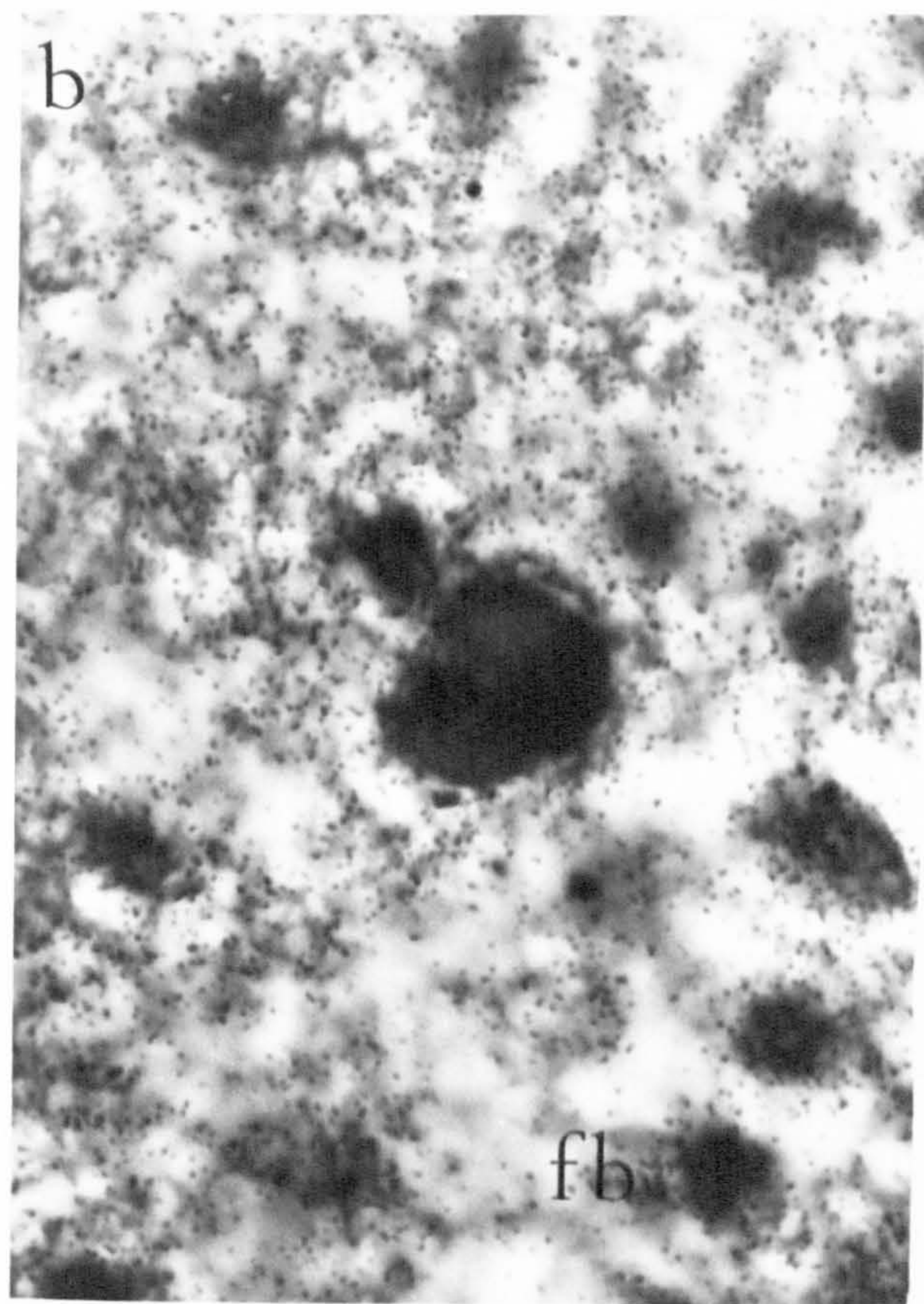
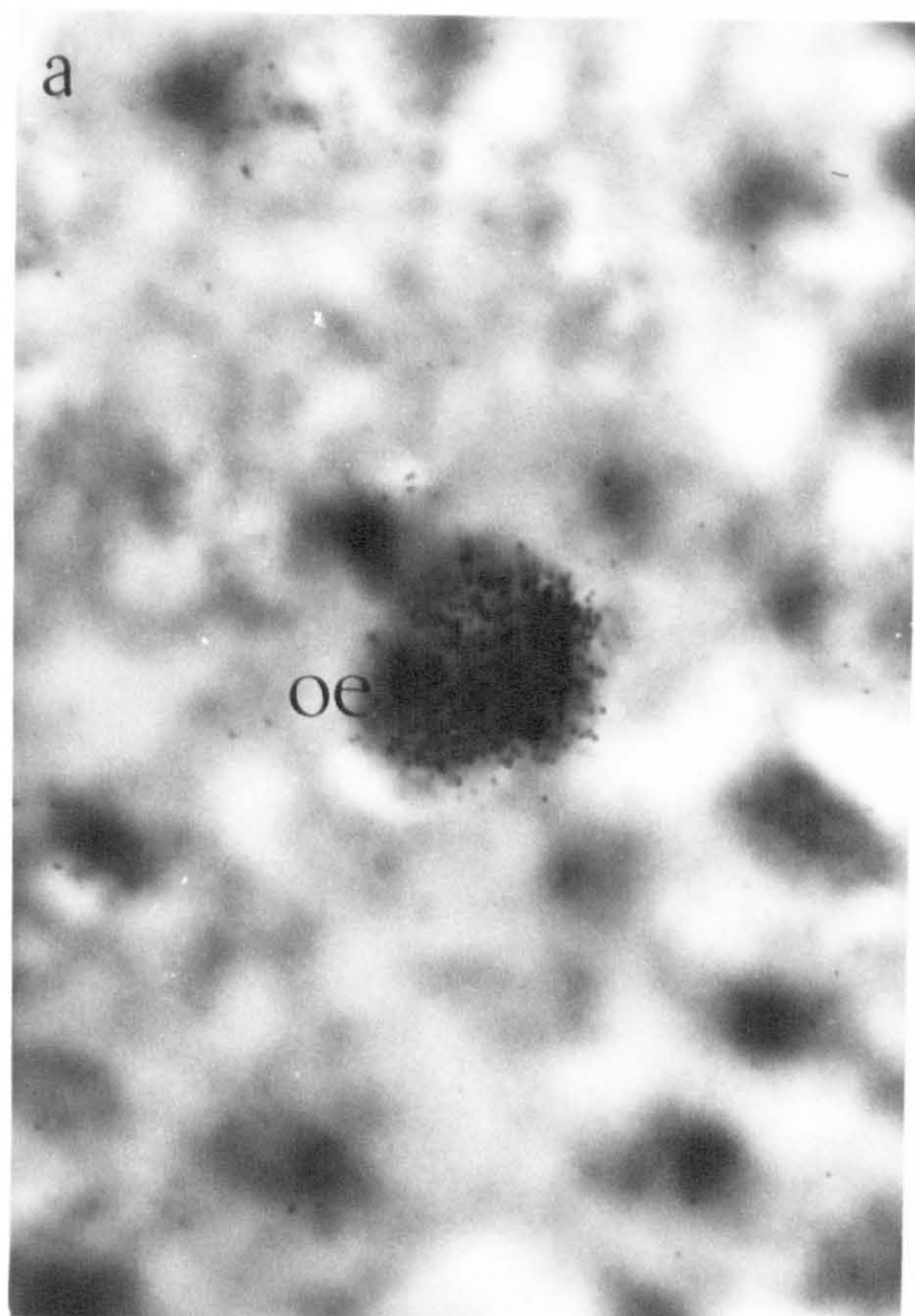
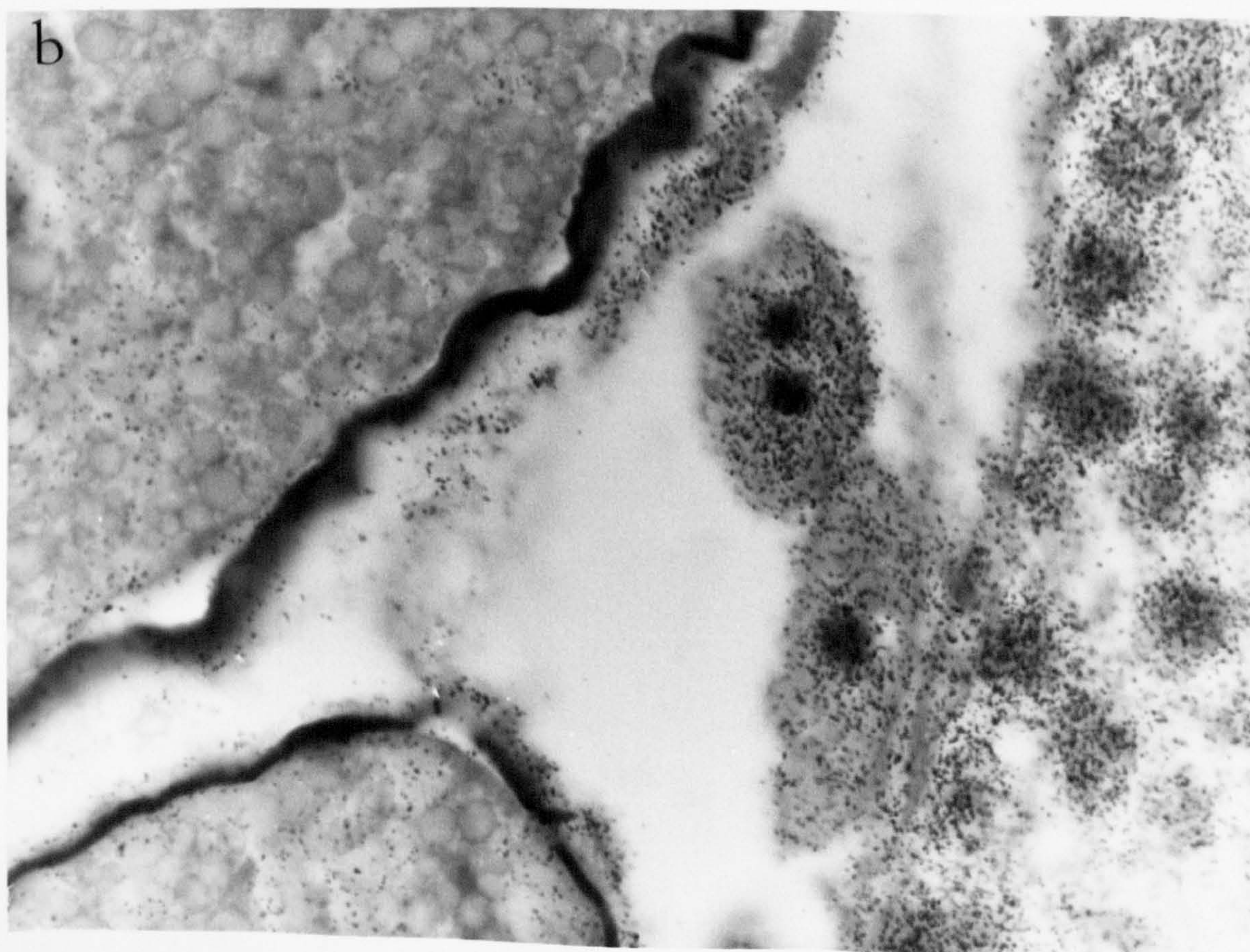
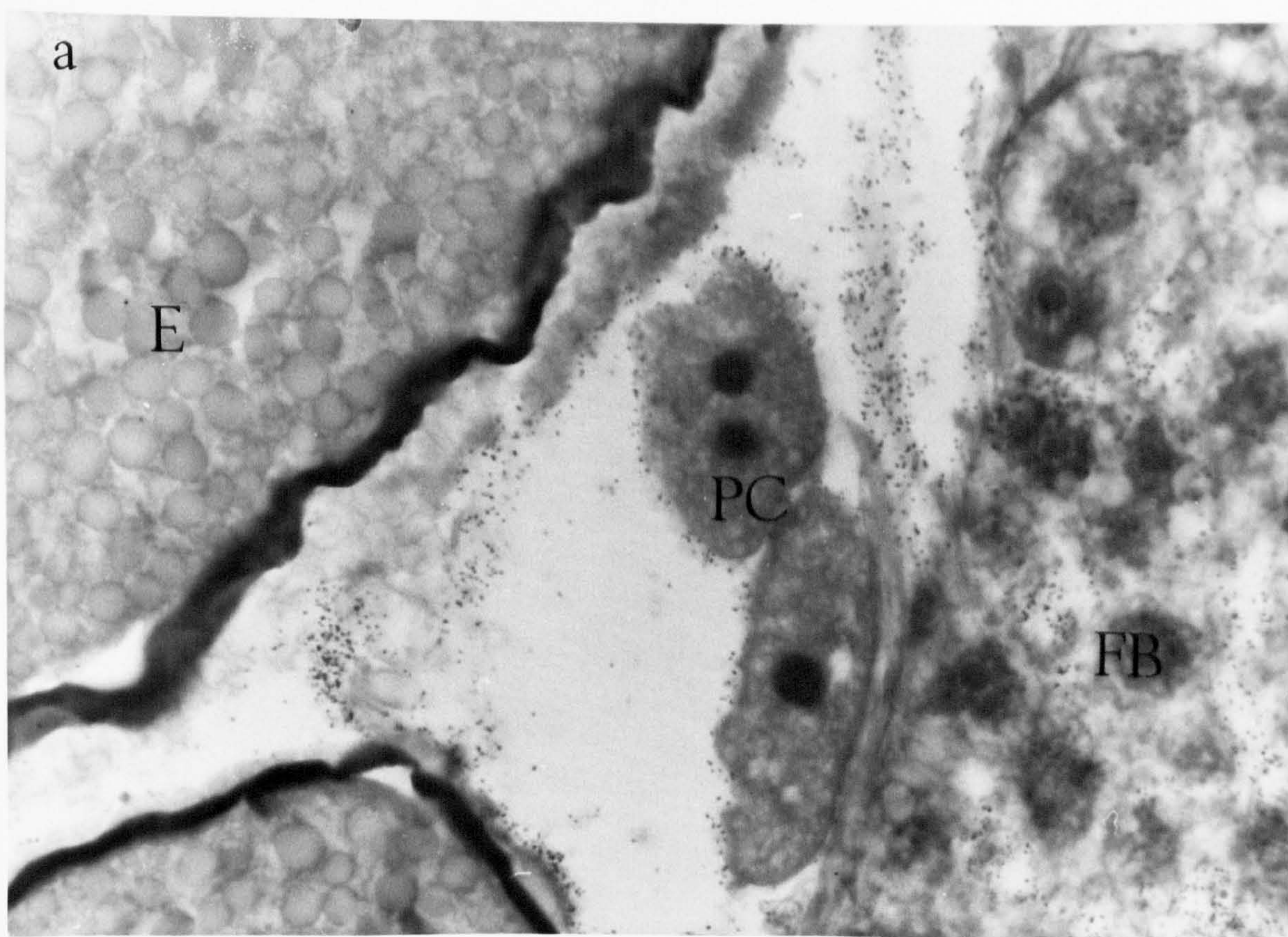


PLATE 10

(a, b) Incorporation of lysine-[H₃] into pericardial cells and fat body of Anopheles labranchiae atroparvus. The isotope was injected at 92 hours pbm. PC = pericardial cells. FB = fat cells. E = current ovarian follicle. In (a) the focus is on the tissues, in (b) on the silver grains.



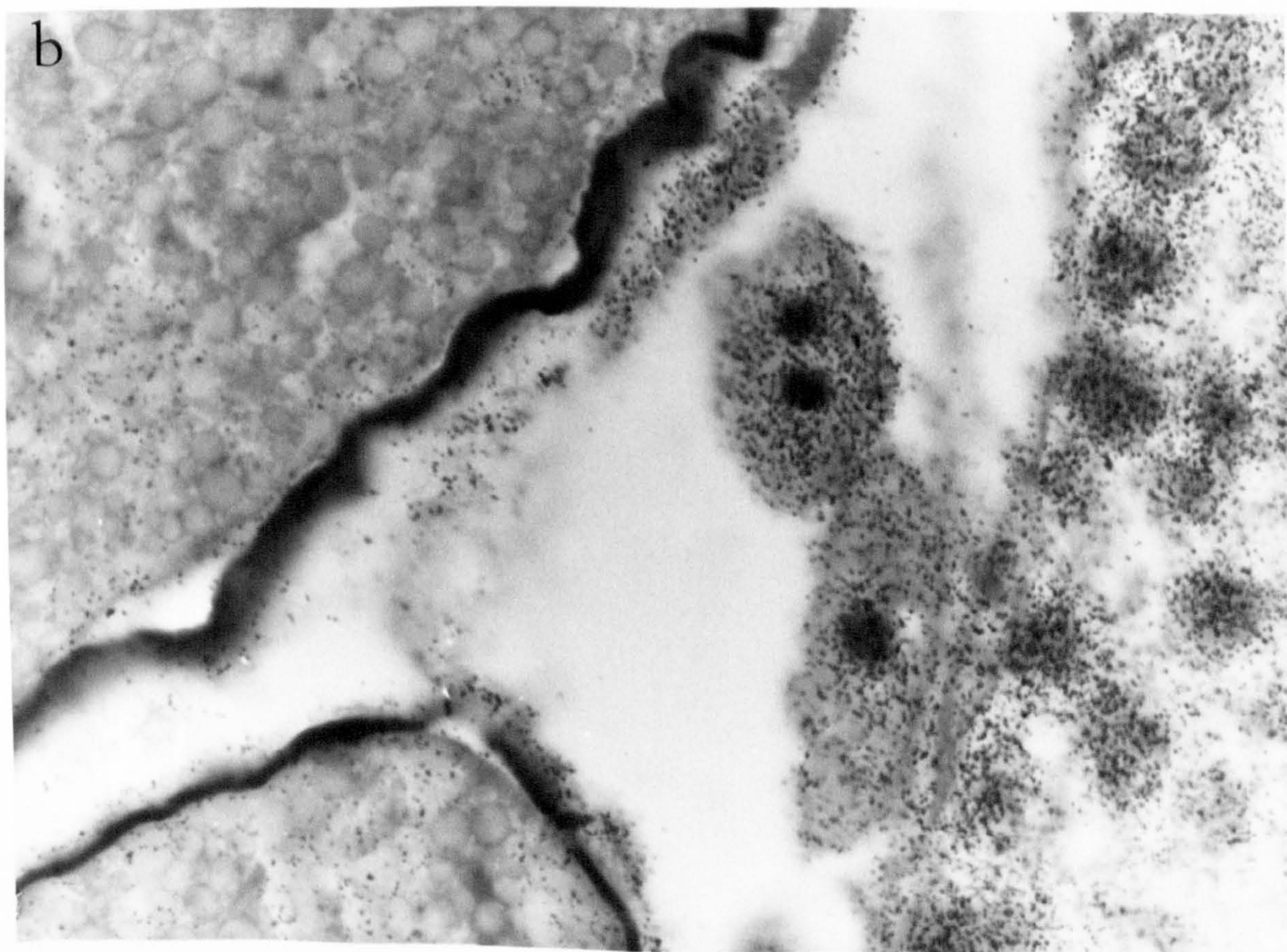
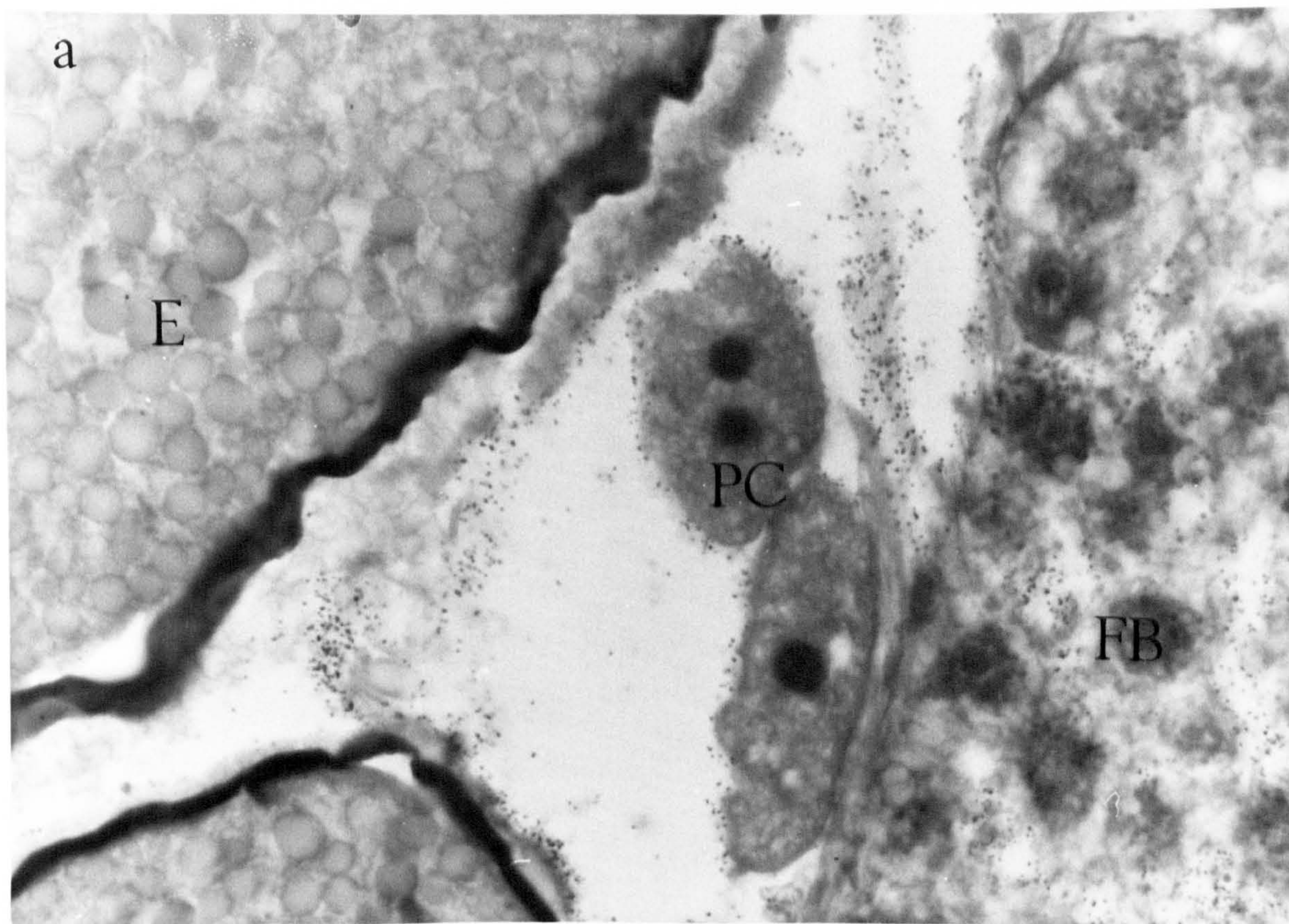


PLATE 11

(a, b) Anopheles labranchiae atroparvus, fixed in Carnoy and stained with Methyl green pyronin. (a) was fixed at 48 hours pbm and shows that the oenocytes stain weakly with pyronin compared to the fat body cells. (b) was fixed at 5 days pbm and shows the reverse of the situation in (a). oe = oenocytes. FB = fat cells. E = current egg follicles, me = midgut epithelium, ml = midgut lumen.

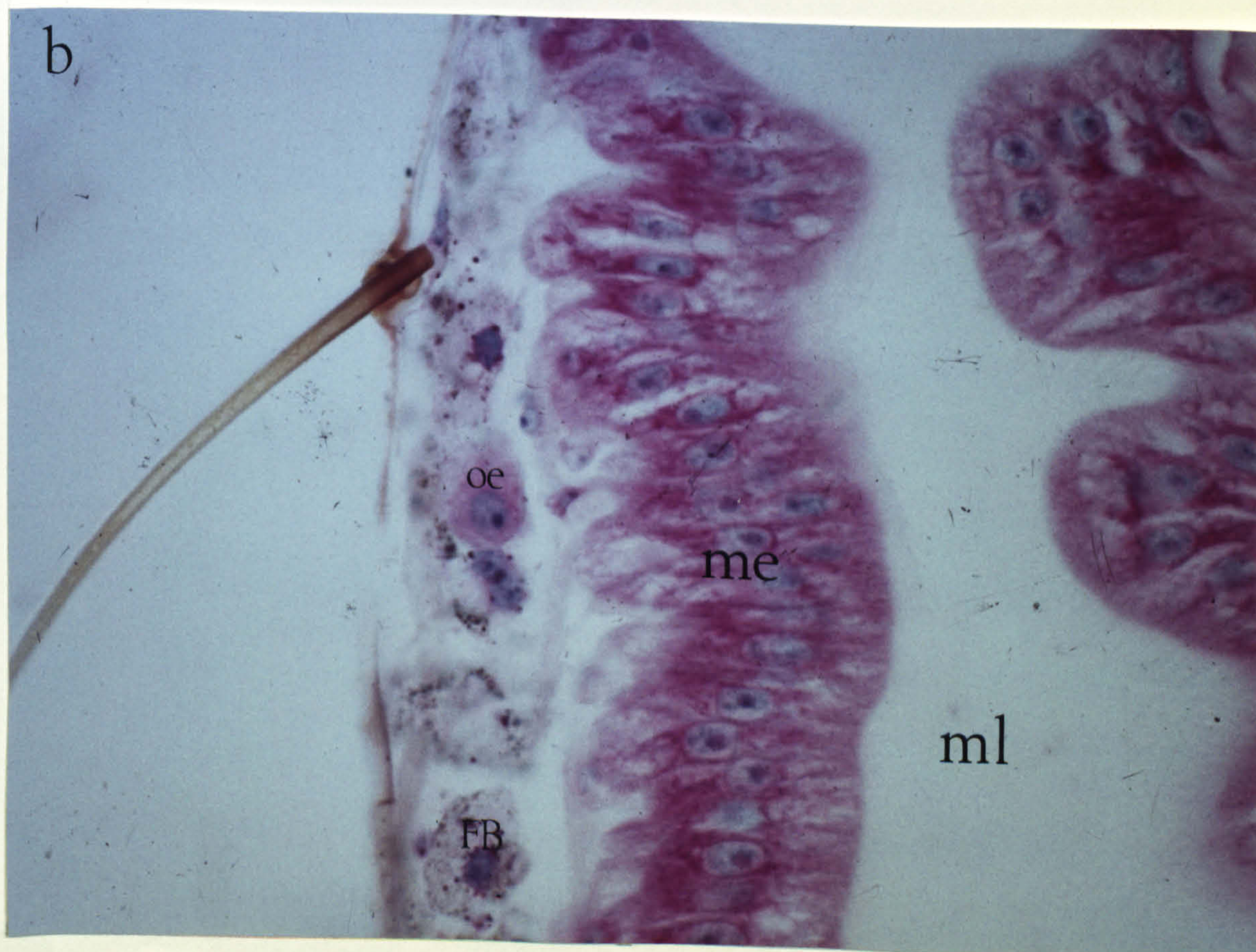
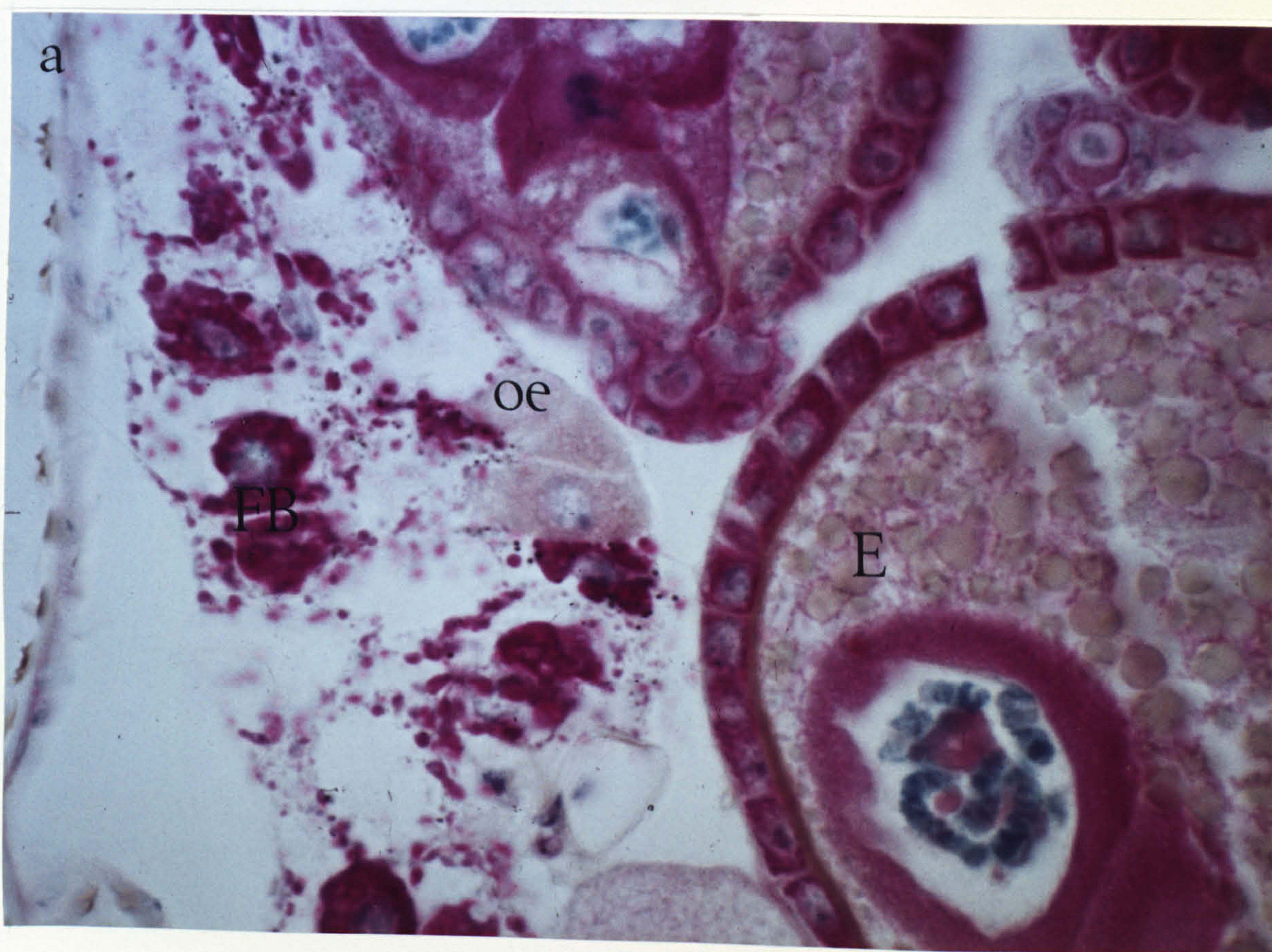
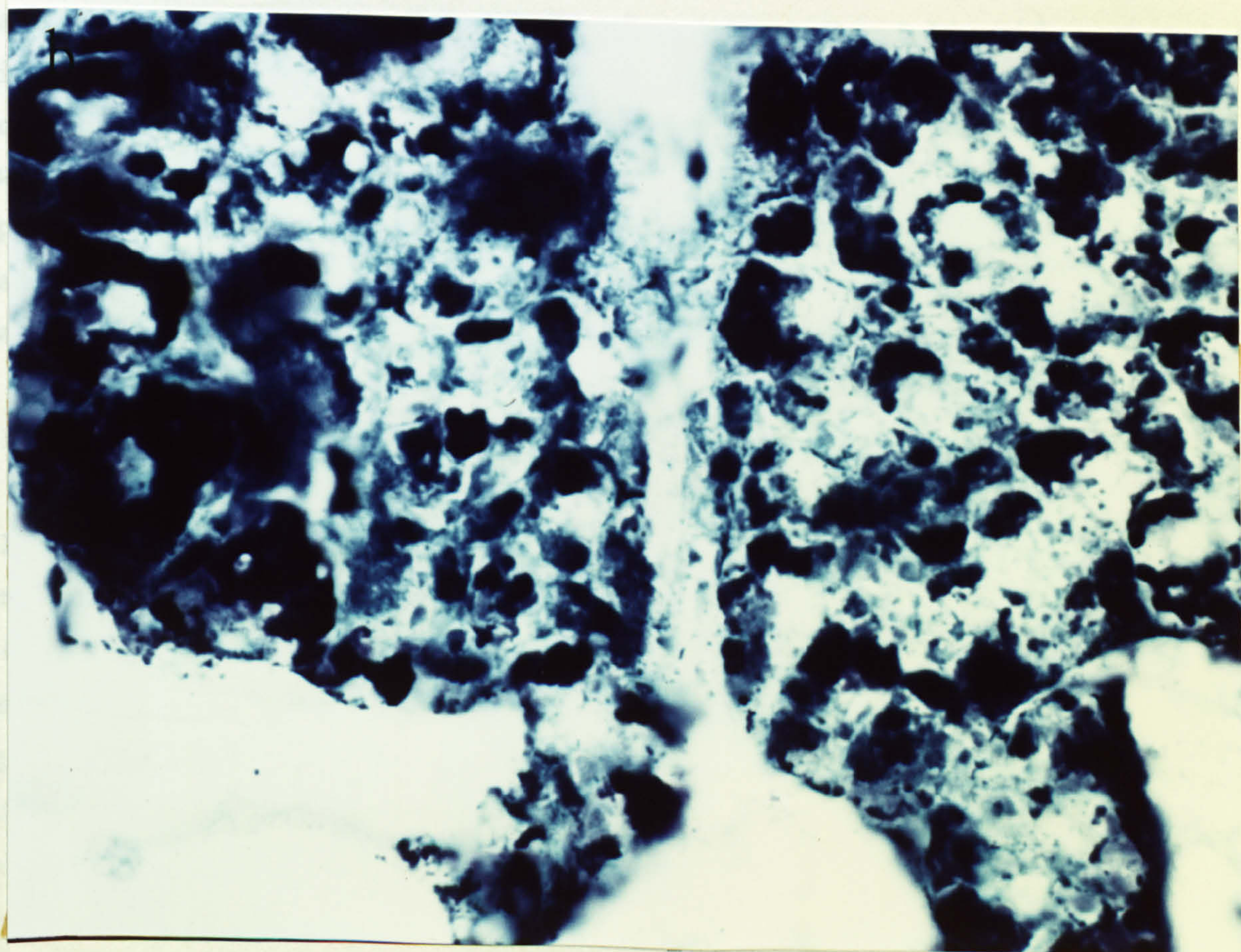
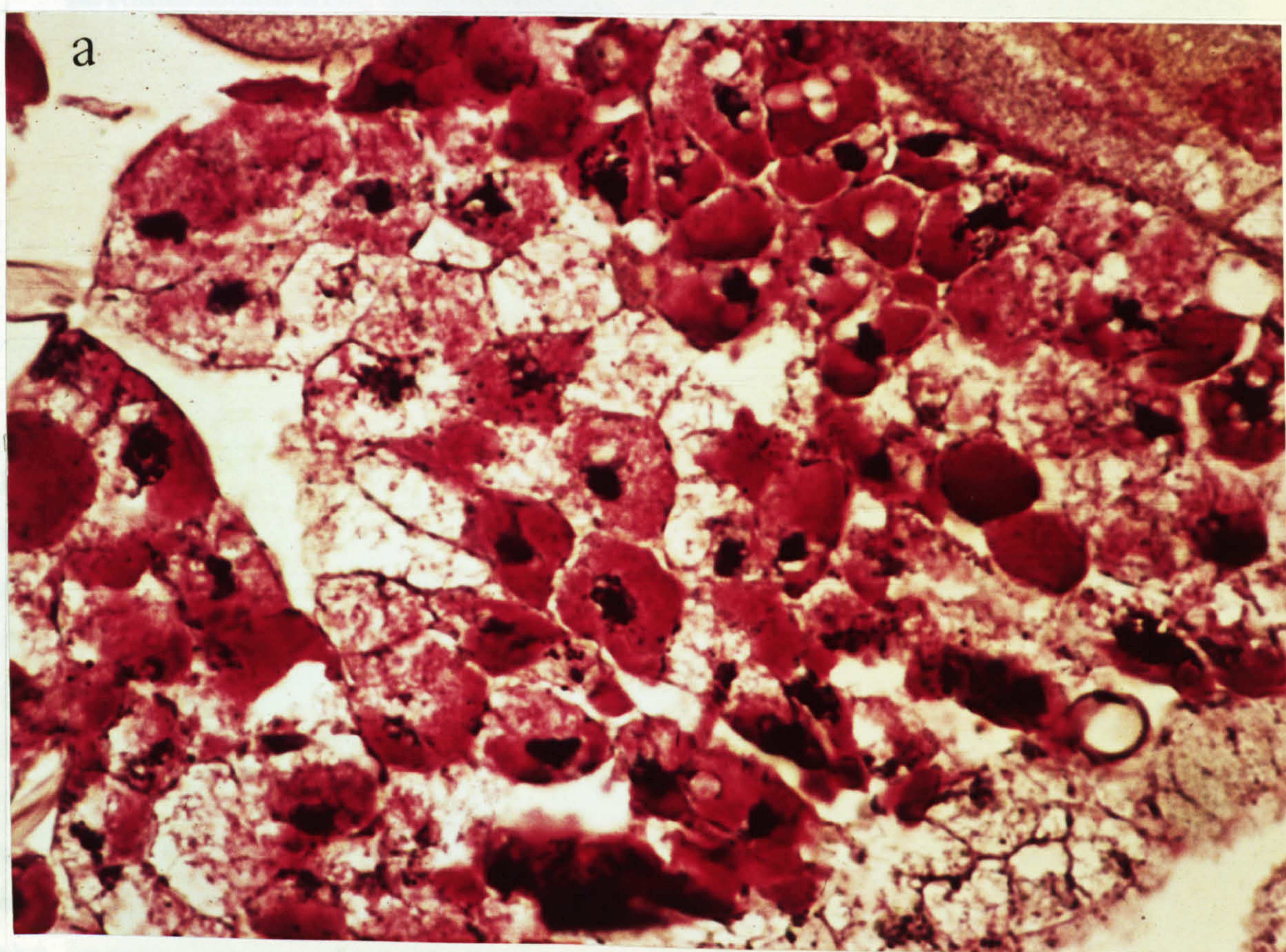


PLATE 12

(a) Fat body of Anopheles labranchiae atroparvus, fixed in FA at 48 hours pbm and stained with PAS.

(b) Fat body of Anopheles labranchiae atroparvus, fixed in FA at 72 hours pbm and stained with Sodium bisulphite toluidine blue.



PART 6

AUTORADIOGRAPHIC, HISTOCHEMICAL AND HISTOPATHOLOGICAL OBSERVATIONS
ON THE MIDGUT, HINDGUT AND MALPIGHIAN TUBULES OF Aedes TOGOI
AND ANOPHELES LABRANCHIAE ATROPARVUS DURING AN INFECTION
WITH BRUGIA PATEI

INTRODUCTION

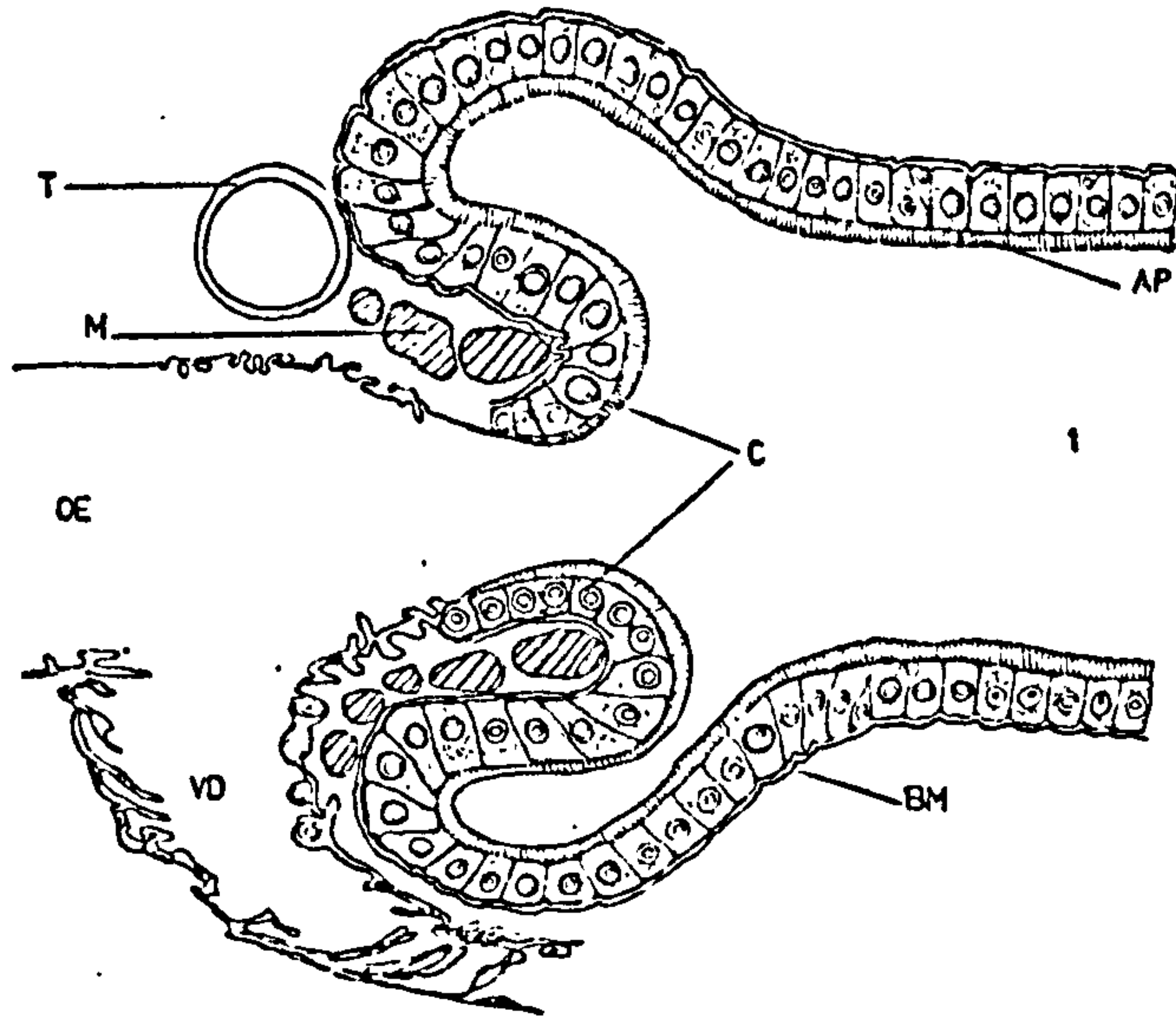
In the adult stage of most mosquito species, a blood meal is necessary to provide a source of protein for the developing eggs (see Part 7) and sugar is needed to provide an energy source for survival and flight (Nayer and Van Handel, 1971; Nayer and Sauerman, 1975b). The digestion of protein and sugar takes place in the midgut (see reviews by Gooding, 1972; 1975; House, 1974). The hindgut is concerned with the selective reabsorption of ions and water from the gut lumen whereas the Malpighian tubules are organs of excretion which filter waste metabolites from the haemolymph (see Berridge, 1970; Madrell, 1971). In this present study the incorporation of radioactive metabolites into the midgut, hindgut and Malpighian tubules of Aedes togoi and Anopheles labranchiae atroparvus is described together with selected histochemical observations of these organs. Also a description is given of the histopathology of the midgut which arises from the ingestion of microfilariae of Brugia patei. To provide an appropriate backcloth for these varied observations the following account outlines the principal histological features of the midgut, hindgut and Malpighian tubules.

1. Midgut

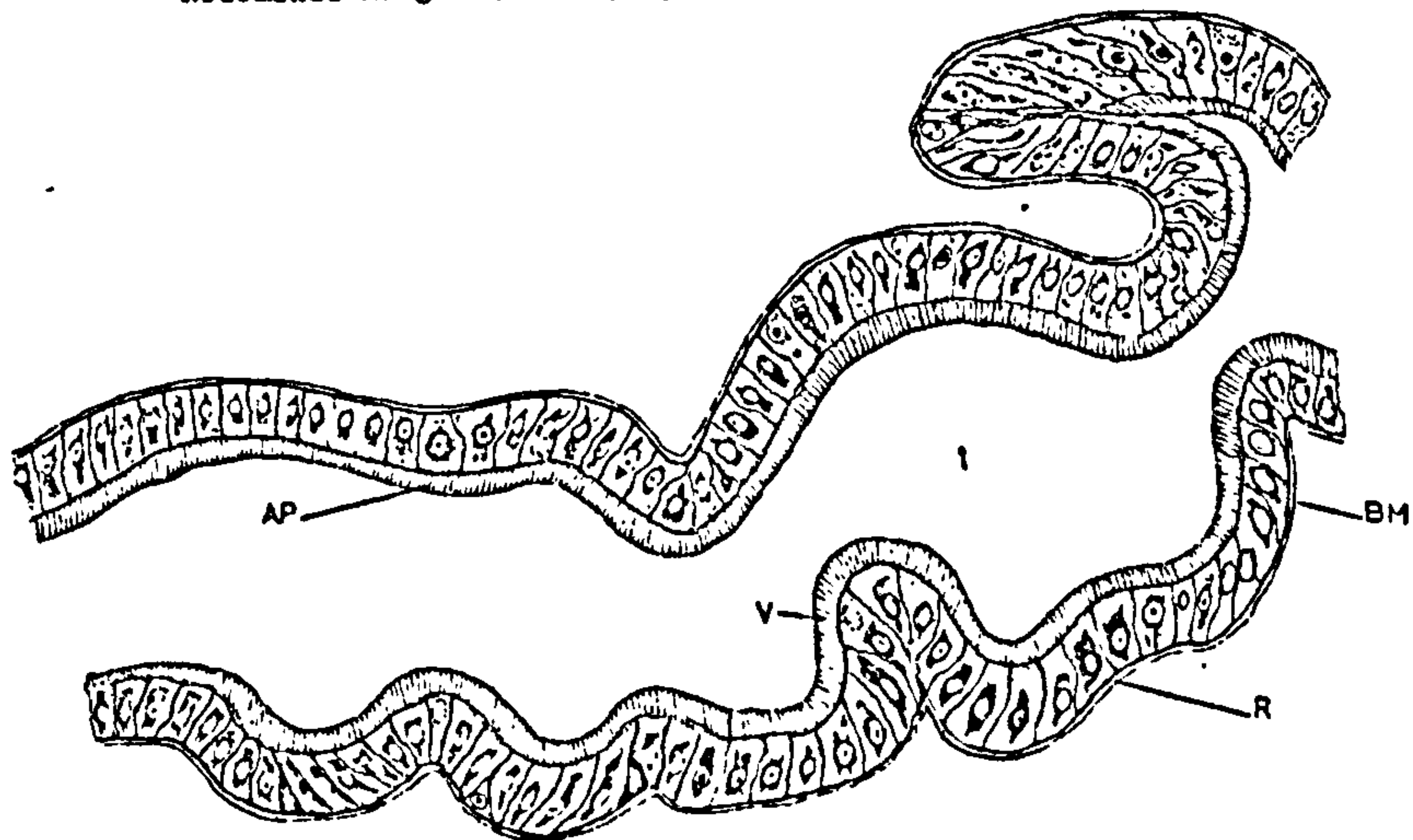
In the thorax the posterior part of the oesophagus is telescoped into the anterior part of the midgut. This region is termed the cardia (Fig 4; Plate 13). Posterior to the cardia the anterior (thoracic) midgut is tubular in shape (Fig 4; Plate 16a) and connects with the posterior (abdominal) midgut, also known as the stomach (Fig 4; Plate 16b). In the unfed female mosquito the wall of the abdominal midgut is thrown into folds, but in the replete, blood-fed female it is balloon shaped (Freyvogel and Staubli, 1965). The wall of the thoracic midgut does not alter its shape when the female takes a blood meal. In structure

Fig 4 Histological features of midgut of *Anopheles labranchiae atroparvus*

Cardia and thoracic midgut (1-5 days pbm).



Abdominal midgut (4-5 days pbm).



c = cardia cells, e = epithelial cells, AP = apical leaflets, BM = basement membrane, R = RNA rich cytoplasm, v = cytoplasmic vacuole, l = gut lumen, VD = lumen of ventral diverticulum, m = muscle, T = trachea.

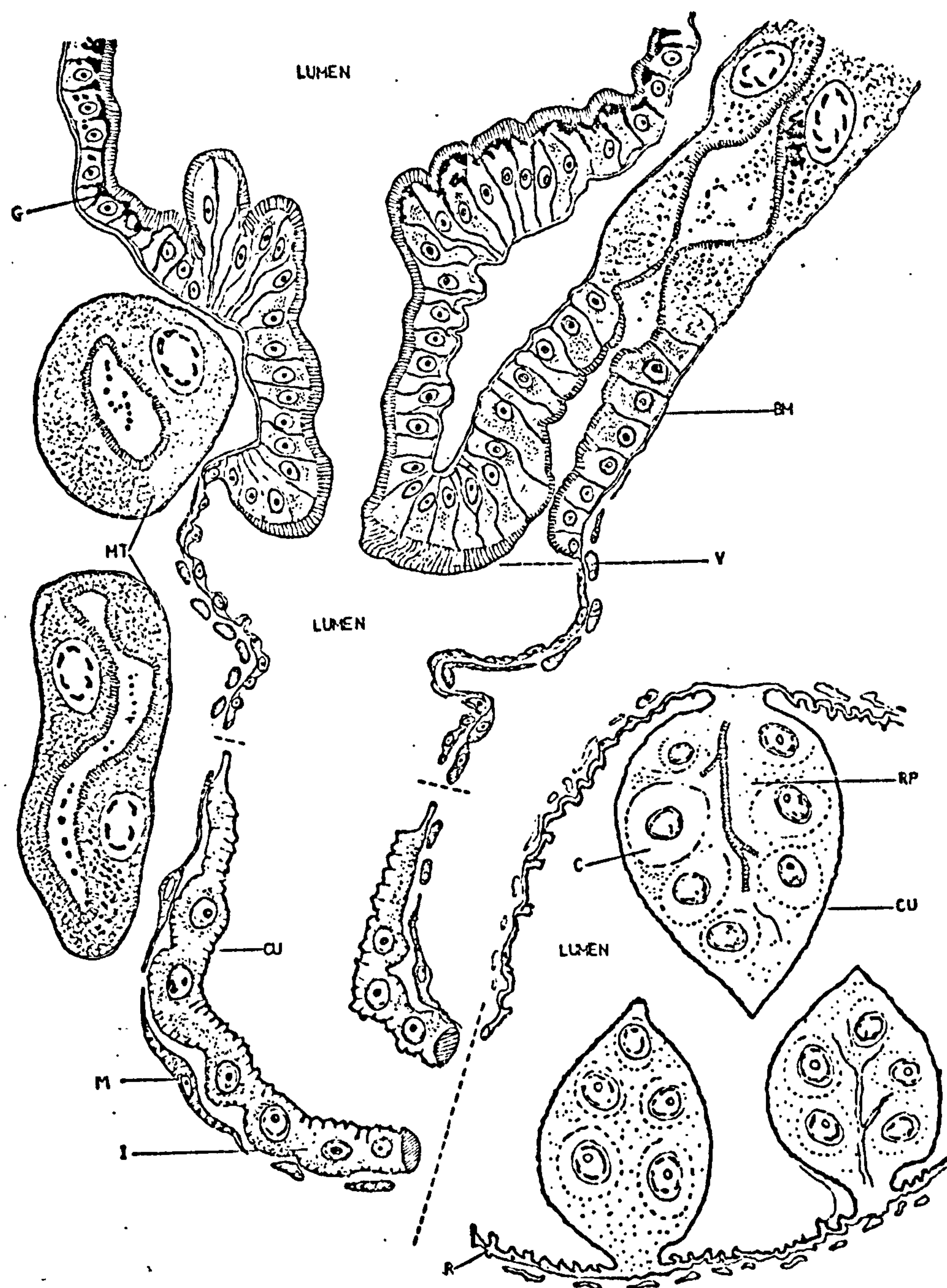
(see Christophers, 1960; Freyvogel and Staubli, 1965) the midgut of the adult female mosquito consists of a single layer of cuboidal to columnar shaped epithelial cells, the luminal surface being modified to form a series of apical leaflets (microvilli) which are recognised under the light microscope as a brush border (Fig 4; Plate 16a). Interspersed among the above typical cells of the midgut are numbers of smaller cells which do not abut onto the gut lumen. These are the basal or nidal cells (Fig 4; Plate 14a) and are thought to be replacement cells for those epithelial cells which get sloughed off into the lumen. The basal surface of the epithelial cells rests upon a structure known to light microscopists as the basement membrane. Ultrastructurally (Bertram and Bird, 1961; Staubli, Freyvogel and Suter, 1966; Hecker et al, 1971; Reinhardt and Hecker, 1973) this is resolved into a basal lamina 300-700Å⁰ thick which is a layer of diffuse material separated from the plasma membrane of the epithelial cells by a clear layer. Collagen fibrils (see review by Ashhurst, 1968; Ashhurst and Costin, 1974) run beneath the basal lamina. The basement membrane is separated from the haemocoel by a thin sheath of muscle cells and tracheole elements. Functionally, the basement membrane may be likened to a filter (Terzakis, 1966).

In blood-fed females, the contents of the blood meal are separated from the epithelial cells of the midgut by a fibrous acellular structure called the peritrophic membrane (Gooding, 1972; Richardson and Romoser, 1972; Romoser and Rothman, 1973; Romoser and Cody, 1975). In Simulium, the peritrophic membrane acts as a barrier to the escape of ingested microfilariae of Onchocerca (Lewis, 1953; Duke and Lewis, 1964; Laurence, 1966) but is ineffective in preventing the escape of microfilariae from the stomach of mosquitoes (Laurence and Pester, 1961; Esslinger, 1962).

2. Hindgut

The hindgut in mosquitoes (Fig 5) is divided into an anteriorly situated ileum (Plates 15b, 17) and a posteriorly placed rectum. Both regions consist of a single layer of epithelial cells, cuboidal in shape in the ileum but flattened and more squamous in the rectum. A prominent feature of the rectum are the rectal papillae, the fine structure of which has

Fig 5 Histological features of hindgut and malpighian tubules of *Anopheles labranchiae atroparvus*



I = ileum, R = rectum, RP = rectal papillae, MT = Malpighian tubules, v = apical leaflets (brush border), cu = cuticular intima, BM = basement membrane, m = muscle, c = cytoplasmic vacuole, g = glycogen.

been described by Hopkins (1967). The basal surface of the epithelial cells of the hindgut rest upon a basement membrane, which together with associated muscle and tracheole elements, separates the epithelial cells from the haemocoel. The apical surface of the epithelial cells is separated from the gut lumen by a thin cuticula intima.

3. Malpighian tubules

At the junction of the midgut and hindgut are the Malpighian tubules (Fig 5). The wall of a Malpighian tubule consists of a single layer of epithelial cells with characteristically large nuclei, enclosing a central lumen which is continuous with that of the midgut. The luminal surface of the tubules is modified to form a series of apical leaflets (brush border). The basal surface of the Malpighian tubules rests on a basement membrane associated with muscle and tracheole elements, but no intima separates the apical leaflets from the lumen of the tubule.

The following observations are based on uninfected as well as infected mosquitoes.

RESULTS

1. Incorporation of radioactive precursors into the midgut, hindgut and Malpighian tubules

(a) Uptake of nucleosides

In female Aedes togoi and Anopheles labranchiae atroparvus there was little incorporation of thymidine-[H₃] and uridine-[H₃] into the epithelial cells of the foregut, hindgut and Malpighian tubules compared to the label recorded over the midgut cells. With thymidine-[H₃], the label was mostly over the nuclei of the basal cells (Plates 13b, 14a). This was so in Aedes togoi that had been injected with the isotope at 3-24 hours pbm, and in Anopheles labranchiae atroparvus that had been injected at 45 hours or 5 days pbm. With uridine-[H₃], the label was centred mainly over the nucleoli of the midgut cells (Plates 13a, 14b). All three divisions of the midgut were labelled as shown for Aedes togoi in Table 20.

(b) Uptake of carbohydrate

Following the injection of tritium labelled monosaccharide, the bulk of the radioactivity was centred over the cardia and thoracic midgut. This is shown for Anopheles labranchiae atroparvus in Table 21. A similar distribution of tritium labelled monosaccharide was recorded for Aedes togoi that had been injected at 4 days pbm. Within the gut epithelial cells, the tritium labelled monosaccharide tended to lie over the apical rather than the basal regions of the cells (Plate 15). In some individuals radioactive material was present in the lumen of the midgut and hindgut.

(c) Uptake of amino acids

The incorporation at 4-5 days pbm of various tritium labelled amino acids into the midgut, hindgut and Malpighian tubules of Aedes togoi and Anopheles labranchiae atroparvus is shown in Table 22. The incorporation at 1-4 days pbm of tritium labelled phenolic amino acids into the gut of Anopheles labranchiae atroparvus is shown in Table 23. With most of the

Table 20 Incorporation of uridine-[H₃] into the midgut of
Aedes togoi parasitised by Brugia pateri

Time injected (pbm)	Mean grain density $\bar{x} \pm$ standard error of mean			
	Cardia	Thoracic midgut	Stomach	Background
26.5 hours	22.0 \pm 2.0	18.28 \pm 1.50	24.68 \pm 3.20	2.03 \pm 0.11
45.0 hours	5.64 \pm 0.75	-	13.28 \pm 6.38	1.79 \pm 0.11
5 days	30.72 \pm 3.60	20.20 \pm 1.90	21.04 \pm 2.50	1.97 \pm 0.14
6 days	33.68 \pm 3.65	35.68 \pm 1.87	58.60 \pm 4.47	1.96 \pm 0.15
8 days	56.96 \pm 5.19	25.08 \pm 1.45	64.88 \pm 7.07	1.97 \pm 0.15

One insect was used at each time interval, and each was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x})
per unit area of tissue = $\frac{\text{(total number grains counted)}}{25 \text{ unit areas/tissue}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{\text{(total number grains counted)}}{100 \text{ unit areas}}$

1 unit area = 64 μm^2 .

- = no observations.

amino acids used, there was little deposition of labelled material into the gut lumen. Exceptions were the phenolic amino acids, and in particular DOPA-[H₃]. Plenty of labelled material was present in the hindgut lumen of Anopheles labranchiae atroparvus that had been injected with this isotope. An interesting finding was that the incorporation of DOPA-[H₃] into the gut of uninfected Anopheles labranchiae atroparvus was higher than that recorded for the gut of individuals infected with Brugia pateri. In the thoracic midgut, the amino acid label was localised over the cytoplasm. Grain counts over the midgut villi were lower than those over the rest of the cell. This difference is illustrated in Plate 16a. In the stomach it was not possible to make a similar distinction (Plate 16b). In the hindgut the amino acid label was often less dense than that over the midgut (Tables 22, 23) and also more diffuse (Plate 17). The amino acid label over the Malpighian tubules was also diffuse.

2. Histochemical observations of the midgut, hindgut and Malpighian tubules

(a) Midgut

Nucleic acids. In the midguts of females fixed in Carnoy at 1-5 days pbm and stained with methyl green pyronin, cytoplasmic pyronin staining was intense throughout this period. Within individuals the cardia cells tended to stain slightly more intensely with pyronin than the epithelial cells of the thoracic and abdominal parts of the midgut. The cytoplasm of the midgut cells was extensively vacuolated and this made it almost impossible to obtain much information on the cytochemical distribution of pyronin in these cells. Pyronin staining was certainly present in the nucleoli and there was a tendency for the cytoplasm in the vicinity of the cell nucleus to be strongly stained with pyronin. This pattern of cytoplasmic staining was more obvious at 4 or more days pbm (see Fig 4b and Plate 11b). The apical leaflets and basement membranes were unstained. Pyronin staining of the midgut cells was prevented if the sections were incubated in ribonuclease prior to staining, indicating that the pyroninophilia was due to RNA.

Carbohydrates. To establish that PAS staining in the midgut was almost certainly due to carbohydrate some sections were acetylated with acetic anhydride in pyridine before performing the PAS step. No PAS staining was present in the acetylated sections. This acetylation blockade was successfully reversed when the sections were saponified in ethanolic KOH and stained with PAS. The restoration of PAS staining by saponification of the acetylated sections indicates that the PAS reactive sites contain carbohydrate. The periodate induced PAS reactivity of the midgut was also prevented when a sodium borohydride or aniline in acetic acid blockade was interposed between periodate oxidation and staining with Schiff's reagent. This suggests that 1:2 glycol groups converted to dialdehydes by periodate oxidation, are responsible for the PAS reactivity. No Schiff's staining was present in unoxidised sections indicating that native aldehydes made no observable contribution towards the PAS reactivity of the sections. 1:2 glycol-rich material that was susceptible to digestion with saliva or amylase solution was considered to be glycogen. The distribution of glycogen in the midgut was also studied by using Best's carmine and the sodium bisulphite-toluidine blue method. With these three techniques it was recorded that glycogen was absent from the midgut of newly emerged females. In blood-fed females no glycogen was observed in the cardia and thoracic midgut, but it was present in the abdominal midgut of females at 1-2 days pbm (Fig 4a), but not in females killed at 3 or more days pbm. Within the midgut cells, glycogen was concentrated in the perinuclear region but was absent from the apical leaflets and midgut lumen. It was also absent from the abdominal midgut cells situated at the junction where the midgut meets the hindgut (Fig 5). Glycogen was usually present in the basement membrane of the midgut. The apical leaflets and basement membrane also contained 1:2 glycol-rich material that was resistant to digestion with saliva or amylase. In addition the apical leaflets contained an Alcian blue positive acid mucosubstance that was unreactive to the diamine methods. As this substance was reactive to both the AB(2.5) and AB(1.0) variants, it evidently contained both carboxylated and sulphated polyanionic groups.

Proteins. The protein detecting methods failed to demonstrate a distinction in staining intensity between the cardia, thoracic and abdominal midgut cells. The cytoplasm of the epithelial cells reacted weakly to these techniques. Tryptophan rich protein was concentrated in

Table 22 - Incorporation of [14 C]-amino acids into the midgut, hindgut and malpighian tubules of *Aedes togoi* and *Anopheles labranchiae atroparvus*

Isotope	Time injected (hours pbm)	Mean grain density \pm standard error of mean				
		Cardia	Thoracic midgut	Abdominal midgut	Hindgut	Malpighian tubules
(a) <i>Aedes togoi</i>						
Histidine	96	6.40 \pm 1.03	4.40 \pm 0.82	8.00 \pm 1.54	4.80 \pm 1.30	4.80 \pm 1.14
Proline	120	5.00 \pm 0.97	2.80 \pm 0.71	2.40 \pm 0.60	1.20 \pm 0.33	0.80 \pm 0.43
Arginine	97	63.60 \pm 5.25	41.80 \pm 2.91	59.0 \pm 6.29	26.0 \pm	35.80 \pm 4.71
Lysine	92	53.20 \pm 1.98	65.20 \pm 3.90	26.40 \pm 3.29	—	14.40 \pm 1.48
Methionine	95	55.20 \pm 2.23	47.60 \pm 2.52	—	20.80 \pm 3.91	24.20 \pm 3.12
Cystine	99	76.20 \pm 2.26	39.20 \pm 3.82	48.40 \pm 5.33	—	17.20 \pm 2.92
Iso-leucine	91	>100	>100	>100	>100	>100
Leucine	93	>100	>100	>100	>100	>100
Valine	90.5	>100	85.0 \pm 1.76	64.40 \pm 2.96	32.60 \pm 3.44	42.40 \pm 1.91
Phenylalanine	92	17.60 \pm 2.19	11.40 \pm 0.92	9.80 \pm 1.03	—	—
Tyrosine	117	30.40 \pm 4.66	22.20 \pm 3.11	28.40 \pm 4.15	7.40 \pm 2.08	15.00 \pm 2.09
Tryptophan	91.5	100	100	87.0 \pm 6.32	26.20 \pm 3.43	56.20 \pm 3.39
(b) <i>Anopheles labranchiae atroparvus</i>						
Histidine	96	72.0 \pm 1.32	10.80 \pm 2.0	6.40 \pm 1.46	9.0 \pm 0.69	4.60 \pm 0.53
Proline	120	11.20 \pm 1.58	8.20 \pm 0.52	7.32 \pm 1.40	8.60 \pm 0.77	4.80 \pm 0.91
Arginine	97	84.60 \pm 2.45	72.40 \pm 2.51	3.40 \pm 4.20	10.40 \pm 0.96	9.60 \pm 2.71
Lysine	92	<100	<100	<100	<100	<100
Methionine	95	76.60 \pm 4.76	77.60 \pm 3.91	99.40 \pm 4.87	53.80 \pm 2.47	34.20 \pm 3.35
Cystine	99	—	—	6.60 \pm 1.31	—	—
Iso-leucine	91	>100	>100	>100	>100	>100
Leucine	93	—	83.0 \pm 8.42	33.20 \pm 4.76	57.80 \pm 4.68	34.60 \pm 2.20
Valine	90.5	>100	>100	>100	>100	>100
Phenylalanine	92	>100	>100	68.0 \pm 3.96	94.0 \pm 2.30	80.2 \pm 2.5
Tyrosine	117	24.40 \pm 0.92	16.40 \pm 3.55	12.60 \pm 2.01	8.80 \pm 1.11	19.20 \pm 2.19
Tryptophan	91.5	82.0 \pm 4.75	69.60 \pm 3.00	25.60 \pm 2.23	62.40 \pm 4.68	27.80 \pm 5.20

Mean grain density (\bar{x}) per unit area of follicular tissue = (total number grains counted) / (number mosquitoes used per tissue x 5 unit areas) One mosquito of each species was used per time interval.

Mean grain density (\bar{x}) per unit area of background = (total number grains counted) / (number mosquitoes used x 100 unit areas) Each mosquito was pulsed for 30 minutes before being killed.

- = no observations. 1 unit area = 64 μ m².

the apical leaflets and disulphide-rich protein in the basement membrane. Not surprisingly the contents of the blood meal stained positively with the protein techniques, particularly with bromphenol blue, so the lack of reactivity of the epithelial cells was not due to a failure of the techniques to work.

Lipids. No Oil red O-stainable material was demonstrated in the cardia and thoracic midgut. However in the abdominal midgut tiny globules of Oil red O-stainable fat were present in the epithelial cells at 24 hours pbm. Some fat was also present in the lumen. At 48 hours pbm no fat was present in the lumen and only traces in the epithelial cells. At 72 hours pbm no fat was seen in the abdominal midgut.

Enzymes. In 24-96 hour blood fed females acid phosphatase activity was localised in the cytoplasm of all three regions of the midgut. The apical leaflets and basement membrane contained no reaction product. Non-specific esterase activity was localised in the cytoplasm of the abdominal midgut cells but not in those of the cardia and thoracic midgut. No reaction product was present in the apical leaflets and basement membrane. β -glucosaminidase activity was localised in the apical leaflets of all parts of the midgut.

(b) Hindgut

Nucleic acids. In the hindguts of females fixed in Carnoy at 1-5 days pbm and stained with methyl green pyronin, cytoplasmic RNA staining tended to be concentrated in the basal regions of the epithelial cells of the ileum but was diffuse in the rectal cells. In the rectal papillae RNA staining was present only as fine, pyronophilic particles scattered around the nuclei.

Carbohydrates. Glycogen was absent from the hindgut of newly emerged and blood fed females. A 1:2 glycol-rich, amylase resistant substance was present in the cuticula intima which lines the hindgut. The intima also contained polyanionic material demonstrable with Alcian blue at pH 2.5 and 1.0 and with LID and HID techniques.

Table 23 Incorporation of phenylalanine-[H₃], tyrosine-[H₃] and DOPA-[H₃] into the gut of
Anopheles labranchiae atroparvus

Isotope	Time of injection (hours pbm)	No mosquitoes used	Mean grain density $\bar{x} \pm$ standard error of mean				
			Cardia	Thoracic midgut	Stomach	Hindgut	Malpighian tubules
Phenylalanine (a)	24	1	41.2 \pm 1.4	40.4 \pm 1.37	49.4 \pm 3.69	30.0 \pm 1.41	28.2 \pm 3.24
	48	1	25.6 \pm 3.03	18.4 \pm 1.72	5.2 \pm 1.19	15.6 \pm 3.82	12.6 \pm 2.17
	72	1	55.40 \pm 8.45	70.40 \pm 7.47	31.40 \pm 3.90	42.80 \pm 6.46	31.40 \pm 3.17
	96	1	<100	<100	<100	<100	<100
Phenylalanine (b)	24	1	65.0 \pm 4.24	64.2 \pm 1.58	63.20 \pm 10.52	48.40 \pm 3.52	40.20 \pm 2.42
	48	1	54.3 \pm 3.0	55.40 \pm 4.09	21.0 \pm 5.72	21.20 \pm 3.44	12.60 \pm 1.20
	72	1	<100	<100	<100	48.20 \pm 1.56	78.0 \pm 9.89
	96	1	<100	<100	<100	<100	72.0 \pm 3.04
Tyrosine	24	3	47.20 \pm 3.22	43.70 \pm 3.27	21.50 \pm 1.91	31.70 \pm 5.82	26.40 \pm 3.76
	48	3	40.30 \pm 2.40	29.90 \pm 3.79	14.0 \pm 1.90	-	-
	72	3	57.20 \pm 2.71	54.60 \pm 1.96	32.93 \pm 8.81	34.73 \pm 4.96	27.33 \pm 2.67
DOPA	24	3	11.30 \pm 2.41	11.20 \pm 2.24	5.90 \pm 1.19	6.0 \pm 0.79	10.2 \pm 1.35
	48	3	4.50 \pm 0.79	6.20 \pm 0.93	3.80 \pm 0.52	11.33 \pm 1.42	7.93 \pm 1.0
	72	3	6.30 \pm 0.97	5.40 \pm 1.26	4.67 \pm 0.05	15.20 \pm 4.55	5.93 \pm 1.02
	96	2	4.70 \pm 0.70	7.60 \pm 0.63	6.53 \pm 0.94	7.30 \pm 0.58	9.93 \pm 1.03

All mosquitoes were pulsed for 30 minutes before killing.

(a) = uninfected individuals.

(b) = infected with *Brugia patei*.

Mean grain density (\bar{x}) = (total number grains counted)
per unit area of gut tissue number mosquitoes used x 5 unit areas/tissue

Mean grain density (\bar{x}) = (total number grains counted)
per unit area of background number mosquitoes used x 100 unit areas

1 unit area = 64 μ m².

- = no observations.

Proteins. The protein detecting methods failed to demonstrate a distinction in staining intensity between the ileum, rectum and rectal papillae. As with the staining of the midgut, the epithelial cells of the hindgut stained weakly for protein. Disulphide-rich protein was present in the cuticula intima and material in the hindgut lumen tended to stain with bromphenol blue.

Lipids and enzymes. In females stained with Oil red O at 1-4 days pbm no neutral fat was detected in the hindgut. The enzymes acid phosphatase, non-specific esterase and β -glucosaminidase also appeared to be absent in blood-fed females at 1-4 days pbm.

(c) Malpighian tubules

Nucleic acids. In the Malpighian tubules of females fixed in Carnoy at 1-5 days pbm and stained with methyl green pyronin, RNA staining of the tubules cytoplasm tended to increase in intensity at increasing times pbm. Within the tubules, the nucleoli were stained prominently with pyronin. The brush border was unstained.

Carbohydrates. The presence of glycogen in the Malpighian tubules was variable even within the tubules of the same individual. However, there was a tendency for the tubules of individuals fixed at 24-48 hours to be packed full with glycogen. At 1:2 glycol-rich, amylase resistant material was present in the basement membrane lining of the tubules. Polyanions were absent.

Proteins. The cytoplasm of the Malpighian tubules stained very weakly with the various protein techniques and the apical leaflets were mostly unstained. An exception was noted when bromphenol blue was used. The apical leaflets and nucleoli stained moderately in comparison to the weak staining of the cytoplasm of the tubule. The lumen of the tubules was generally devoid of stainable material but in newly emerged individuals, a cationic (reactive to acid dyes) substance was sometimes present.

Lipids. In females stained with Oil red O at 1-4 days pbm neutral fat was present in the cytoplasm of the tubules up to 3 days pbm. Within an individual the tubules varied considerably in their content of Oil red O

Table 24 Histochemistry of midgut, hindgut and Malpighian tubules of *Anopheles labranchiae atroparvus*

Technique	Midgut						Hindgut				Malpighian tubules	
	cardia		thoracic	abdominal		ileum		rectum		cyt	apical leaflets	
	cyt	cyt	apical leaflets	cyt	apical leaflets	BM	cyt	intima	cyt			intima
<u>Nucleic acids</u>												
Methyl green pylonin	+++	+++	-	+++	-	-	++	-	++	-	++	-
Ribonuclease control	-	-	-	-	-	-	-	-	-	-	-	-
<u>Carbohydrates*</u>												
Best's carmine	-	-	-	++	-	-	-	-	-	-	-	-
Amylase control	-	-	-	-	-	-	-	-	-	-	-	-
Sodium bisulphite toluidine blue	-	-	-	++	-	-	-	-	-	-	++	+-
Amylase control	-	-	-	-	-	-	-	-	-	-	-	-
PAS	-	-	-	++	-	+++	-	+	-	+	+++	+-
Amylase control	-	-	-	-	-	-	-	-	-	-	-	-
Acetylation-PAS	-	-	-	-	-	-	-	-	-	-	-	-
Acetylation-saponification + PAS	-	-	-	+	-	++	-	+	-	+	++	-
PAS with aldehyde blockade	-	-	-	-	-	-	-	-	-	-	-	-
AB(2.5)	-	-	+	-	+	-	-	++	-	++	-	-
AB(1.0)	-	-	+	-	+	-	-	+	-	+	-	-
LID	-	-	-	-	-	-	-	++	-	++	-	-
HID	-	-	-	-	-	-	-	++	-	++	-	-
AB (CEC)	-	-	-	-	-	-	-	+	-	+	-	-
0.6M	-	-	-	-	-	-	-	+	-	+	-	-
1.0M	-	-	-	-	-	-	-	+	-	++	-	-
<u>Proteins</u>												
Bromphenol blue	+	+	+	+	+	-	+	++	+	++	+	+
DMAB-nitrite	+-	+-	+-	+-	+-	-	+-	+-	+-	+-	+-	+-
Peracetic acid-AB	-	-	+	-	+	+++	-	+++	-	+++	-	-
Mercury-orange	-	-	-	-	-	-	-	-	-	-	-	-
Diazo-coupling	+	+	+	+	+	+	+	+	+	+	+	+
Millons	-	-	-	-	-	-	-	-	-	-	-	-
Mixed anhydride	-	-	-	-	-	-	-	-	-	-	-	-
Massons fontana	-	-	-	-	-	-	-	-	-	-	-	-
<u>Lipids</u>												
Oil red O	-	-	-	++	-	-	-	-	-	-	+++	-
Copper pthalocyanin	+	+	+	+	+	-	+	+	+	+	+	+
<u>Enzymes</u>												
Acid phosphatase	++	++	-	++	-	-	-	-	-	-	++	-
β-glucosaminidase	-	-	+	-	+	-	-	-	-	-	-	-
Non-specific esterase	-	-	-	++	-	-	-	-	-	-	-	-

Intense staining = +++, moderate staining = ++, positive staining = +, equivocal staining = +-, negative staining = -. Cyt = cytoplasm, BM = basement membrane of gut. A blank space in the table indicated that no observations of the tissue were made at that particular time interval.

* Glycogen present in abdominal midgut only during the first two days after the blood meal.

** Fat present in abdominal midgut and Malpighian tubules only during the first two days after the blood meal.

stainable fat, some being packed, others empty of fat.

Enzymes. In 24-96 hour blood fed females, acid phosphatase activity was present in the cytoplasm of the tubules, but the enzymes non-specific esterase and β -glucosaminidase appeared to be absent.

With respect to the nucleic acid, carbohydrate and protein histochemical results, the presence of normal or abnormal larvae of Brugia patei appeared to have no affect on the staining patterns. Only a few parasitised individuals were studied with oil red O, and only uninfected individuals examined for enzyme histochemistry. The histochemical findings are summarised in Table 24.

3. Histopathology

Aedes togoi and Anopheles labranchiae atroparvus infected with Brugia patei were fixed in FA immediately after the infective feed and subsequently at intervals until 6 hours later. Individuals were sectioned serially and stained with either CHP or H and E. The midguts of the infected individuals were compared histologically with those of uninfected controls.

Necrotic lesions were observed only in the abdominal midgut of the infected individuals and tended to occur more frequently in the region where the abdominal midgut joins the hindgut. The cytoplasm of the affected cells contained small holes and was intensely acidophilic (Plate 18). The nuclei of some of these damaged epithelial cells was pycnotic. It is suggested that these areas of focal epithelial cell necrosis represent damage caused by the migration of microfilariae out of the midgut. No necrotic lesions were recorded in the midgut of individuals killed at 24 hours pbm or at later time intervals. This suggests that the damaged cells either recover or are replaced.

DISCUSSION

In Aedes togoi intense thymidine [H_3] incorporation into the midgut epithelium was observed in individuals that had been injected between 3-24 hours, or at 5 days pbm, and a similar pattern of thymidine- $[H_3]$ incorporation was recorded in the gut epithelium of Anopheles labranchiae atroparvus at 5 days pbm. The significance of this labelling remains unknown. It is definitely not related to any mitotic activity of the midgut cells, as no mitoses have been seen in the midgut epithelium of the adult female in spite of the fact that over 2000 serially sectioned adults have been examined histologically (see also Treager, 1937). In comparison mitotic figures are readily seen in the follicular epithelium of the developing ovarian follicles (Laurence and Simpson, 1974).

Following an infective blood meal the majority of filarial larvae that are able to migrate through the wall of the abdominal midgut, do so within the period 0-6 hours pbm (Laurence and Pester, 1961). In Aedes togoi and Anopheles labranchiae atroparvus, microfilariae of Brugia patei produce lesions in the wall of the abdominal midgut which are readily observed histologically. The lesions were present in individuals fixed within the period 0-6 hours pbm but were absent in individuals that had been fixed at 24 hours pbm or even later. It is concluded that either the damaged epithelial cells recover, or they are sloughed off into the gut lumen, the gaps in the epithelial lining being filled by enlargement of the basal cells. It is the latter, which in the main, incorporate thymidine- $[H_3]$. Nucleic acid synthesis in relation to wound repair is known to occur in some insects (see Berry et al, 1967). In Aedes aegypti, healing of wounds made by needle puncture of the replete female midgut was slow, some wounds remaining unhealed at 24-48 hours (Day and Bennetts, 1953), whereas in Aedes togoi and Anopheles labranchiae atroparvus, the wounds made by the migrating larvae of Brugia patei were small and evidently healed quickly. Consequently it is most unlikely that thymidine- $[H_3]$ marking of the midgut epithelium in individuals fixed at 5 days pbm represents part of a repair response, particularly as the midgut of filarially infected mosquitoes appeared normal by 24 hours pbm. In Aedes aegypti parasitised by Brugia pahangi, Omar and Gwadz (1974) suggested that the pattern of differential thymidine- $[H_3]$ incorporation

into the midgut epithelium was related to an uneven metabolic activity of the cells as a result of the blood meal. There was little incorporation of thymidine- $[H_3]$ by the midgut epithelium of non-blood-fed controls. It is probable that the basal cells of the mosquito midgut epithelium undergo continual DNA turnover, the level of which rises during the period of blood meal digestion.

In Aedes togoi and Anopheles labranchiae atroparvus all three regions of the midgut incorporated uridine- $[H_3]$. In Aedes togoi incorporation of uridine- $[H_3]$ into the midgut epithelium continues throughout the period 1-8 days pbm. Both within and between individuals there was marked variation in label over the cardia, thoracic and abdominal midgut cells suggesting that there is an uneven pattern of RNA synthesis in the midgut of Aedes togoi both during and well after the digestion of the blood meal. However, this variation in RNA synthesis is not great enough to be detected by qualitative histochemical techniques. RNA staining of the midgut epithelium was intense throughout the period 1-5 days pbm.

Compared to most other tissues, the midgut epithelium of blood-fed Aedes togoi and Anopheles labranchiae atroparvus showed a marked incorporation of glucose- $[H_3]$, galactose- $[H_3]$ and glucosamine- $[H_3]$. The carbohydrate label was comparatively dense over the epithelium of the cardia and thoracic midgut in relation to that recorded over the abdominal midgut. It is suggested that this differential pattern of carbohydrate incorporation hints at a functional distinction between the anterior and posterior parts of the midgut. In the female mosquito the taking of a blood meal at regular intervals is an act closely integrated into the mechanics of egg development (described next in Part 7). Conversely, the taking of a sugar meal, although essential for the survival of the individual (Nayer and Sauerman, 1974a) and as a source of flight energy (Clements, 1955; Nayer and Van Handel, 1971) does not appear to follow a regular pattern. In mosquitoes the contents of a sugar meal are passed to the diverticula for storage, whereas the contents of a blood meal go direct to the abdominal midgut (Trembley, 1952; Day, 1954; Gooding, 1972). The diverticula presumably release the carbohydrate into the midgut as and when required. The utilisation of carbohydrate occurs very quickly. Nayer and Van Handel (1971) found that mosquitoes flown to exhaustion could resume active flight immediately after being given a

sugar meal. In unfed individuals, sustained flight is possible at the expense of glycogen stores in the fat body (Clements, 1955). It is suggested that rapid absorption of carbohydrate entering the midgut occurs in the cardia and thoracic midgut, thus leaving the abdominal midgut free to deal separately with the essentially protein constituents of the blood meal. The preferential incorporation of radioactive carbohydrate from the haemolymph into the anterior midgut can be explained by postulating a rapid absorptive capacity for the latter.

The midgut epithelial cells readily incorporated labelled amino acids from the haemolymph. There was no distinction in relative uptake between the cardia, thoracic and abdominal midgut. The amino acid labelling of the midgut cells may reflect a demand for a steady supply of precursors necessary for the synthesis of new enzymes to replace those released into the midgut lumen after sugar or blood feeding. Information on the types of enzymes and their spatial distribution in the mosquito midgut is scanty. In Aedes togoi and Anopheles labranchiae atroparvus acid phosphatase activity is present in the cytoplasm of all parts of the midgut, whereas non-specific esterase activity is present in the cytoplasm of only the abdominal midgut cells. β -glucosaminidase activity is present in the apical leaflets of the midgut. Biochemical studies have shown that there is a change in proteolytic and non-specific enzyme activity of the midgut after a blood meal (Fisk and Shambaugh, 1952; Gooding, 1966; Briegel and Freyvogel, 1973). A triglyceride-splitting esterase has been identified in the midgut of both sugar and blood fed Aedes aegypti (Geering and Freyvogel, 1975). In Aedes togoi and Anopheles labranchiae atroparvus, neutral fat and glycogen accumulate in the cytoplasm of the abdominal midgut cells up to 48 hours after the blood meal. In comparison no neutral fat or glycogen was observed in the epithelial cells of the cardia and thoracic midgut.

There was some incorporation of labelled nucleosides, carbohydrates and amino acids into the hindgut and Malpighian tubules of blood fed Aedes togoi and Anopheles labranchiae atroparvus. Radioactive material was often present in the lumen of these organs. The nature of this material is unknown but work on other insects has shown that amino acids may be a component of the primary filtrate produced by the Malpighian tubules (Maddrell, 1971) and these may be reabsorbed in the hindgut (Cochran, 1975).

It is probable that excess sugars in the haemolymph are also excreted by the Malpighian tubules. Glycogen is commonly present in the Malpighian tubules of blood fed Aedes togoi and Anopheles labranchiae atroparvus so it is also probable that some of the excess sugars removed by the tubules are reclaimed and stored by undergoing conversion to glycogen.

In conclusion these autoradiographic and histochemical observations, although incomplete, still manage to convey the fact that during the first 4-5 days after blood feeding, intense metabolic activity is centred over the midgut. The activity of the latter, together with that of the fat body already considered in Part 5, is geared to the problem of producing a batch of ripe eggs. The developing filarial larvae compete for the same pool of metabolites during this period also. Autoradiographic and histochemical observations of egg development are considered next in Part 7.

PLATE 13

(a) Incorporation of uridine-[H₃] into cardia of Aedes togoi. The isotope was injected at 5 days pbm.

(b) Incorporation of thymidine-[H₃] into thoracic midgut of Aedes togoi. The isotope was injected at 3 hours pbm. Note that the basal cells (bc) show intense thymidine-[H₃] labelling.

a



b

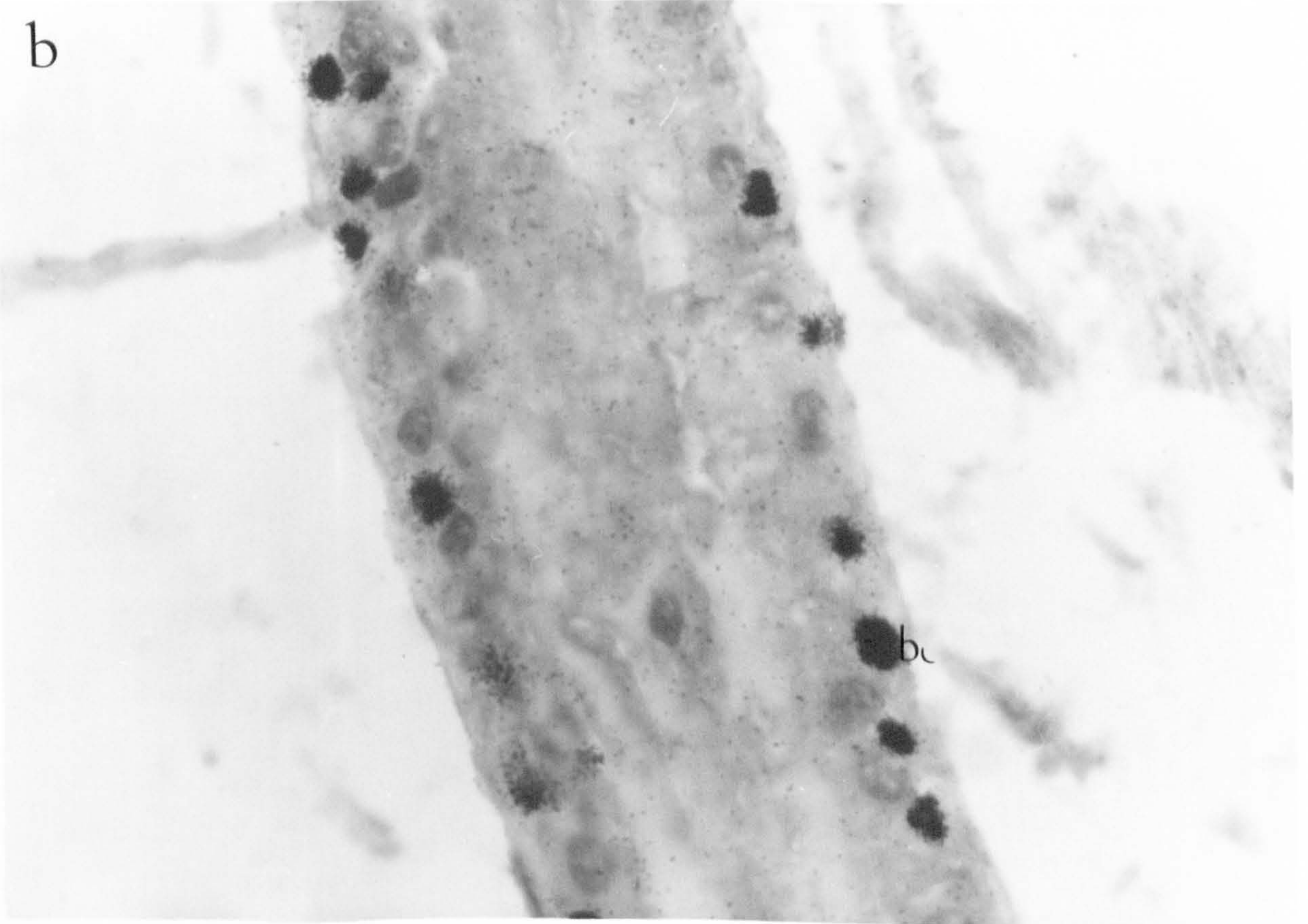


PLATE 14

(a) Incorporation of thymidine-[H₃] into basal cells of abdominal midgut of Aedes togoi. The isotope was injected at 9 hours pbm. bc = basal cell. ml = midgut lumen.

(b) Incorporation of uridine-[H₃] into abdominal midgut of Aedes togoi. The isotope was injected at 6 days pbm. ml = midgut lumen.

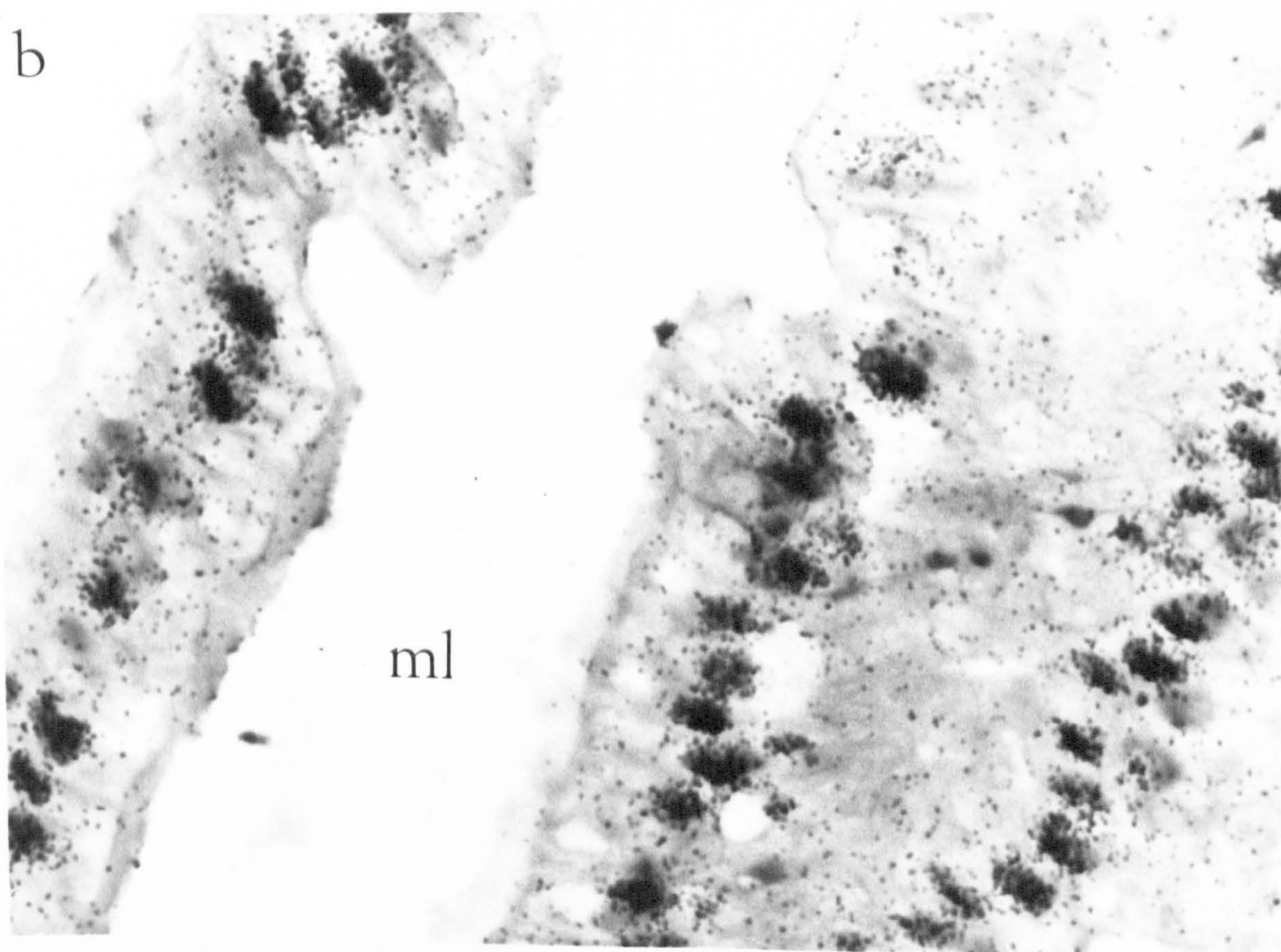
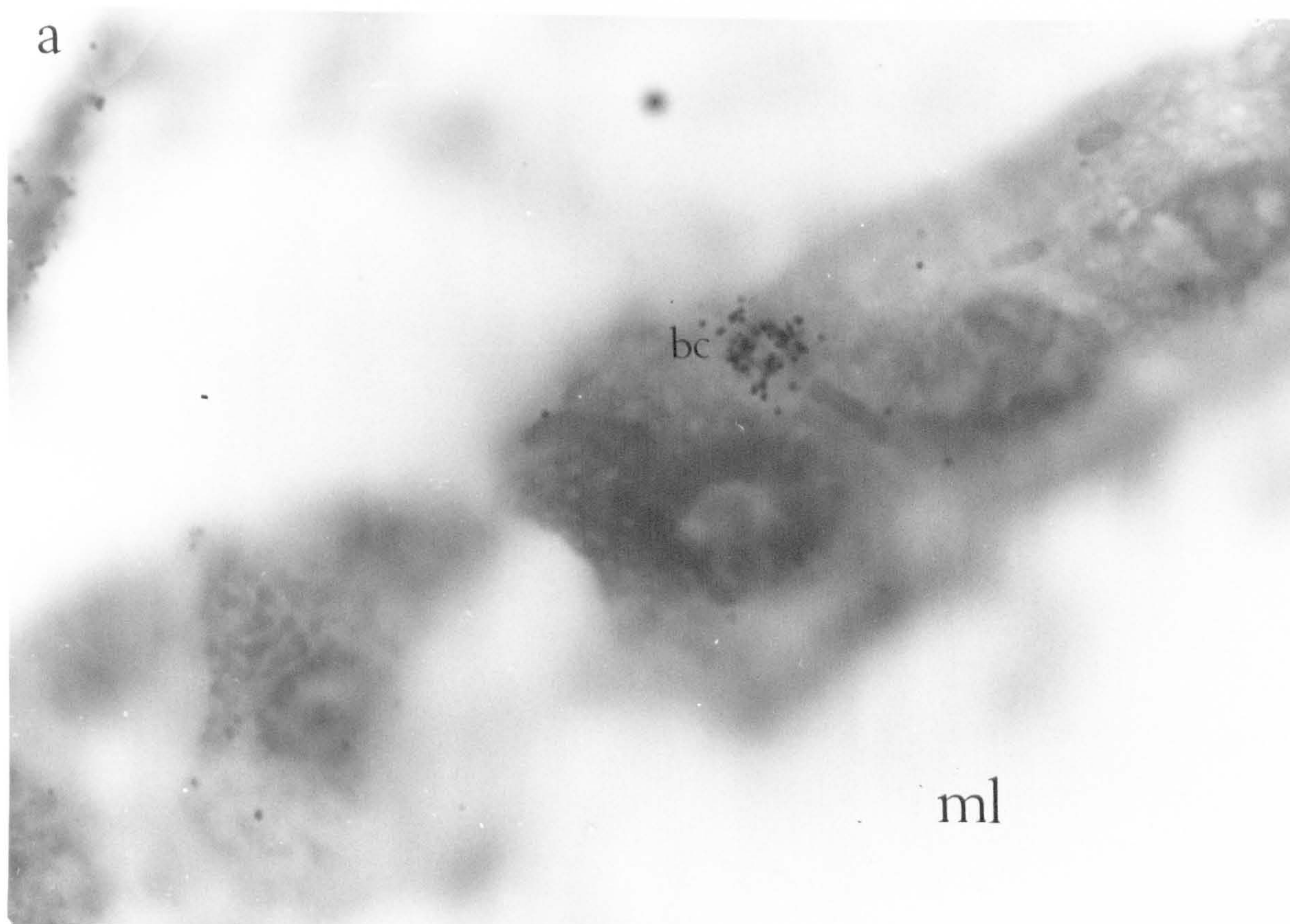


PLATE 15

(a) Incorporation of glucose into thoracic midgut of Anopheles labranchiae atroparvus. The isotope was injected at 72 hours pbm. ml = midgut lumen.

(b) Incorporation of glucose-[H₃] into ileum of Anopheles labranchiae atroparvus. The isotope was injected at 24 hours pbm. l = lumen of ileum. Note that the silver grains are predominantly located at the apical surface of the cells.

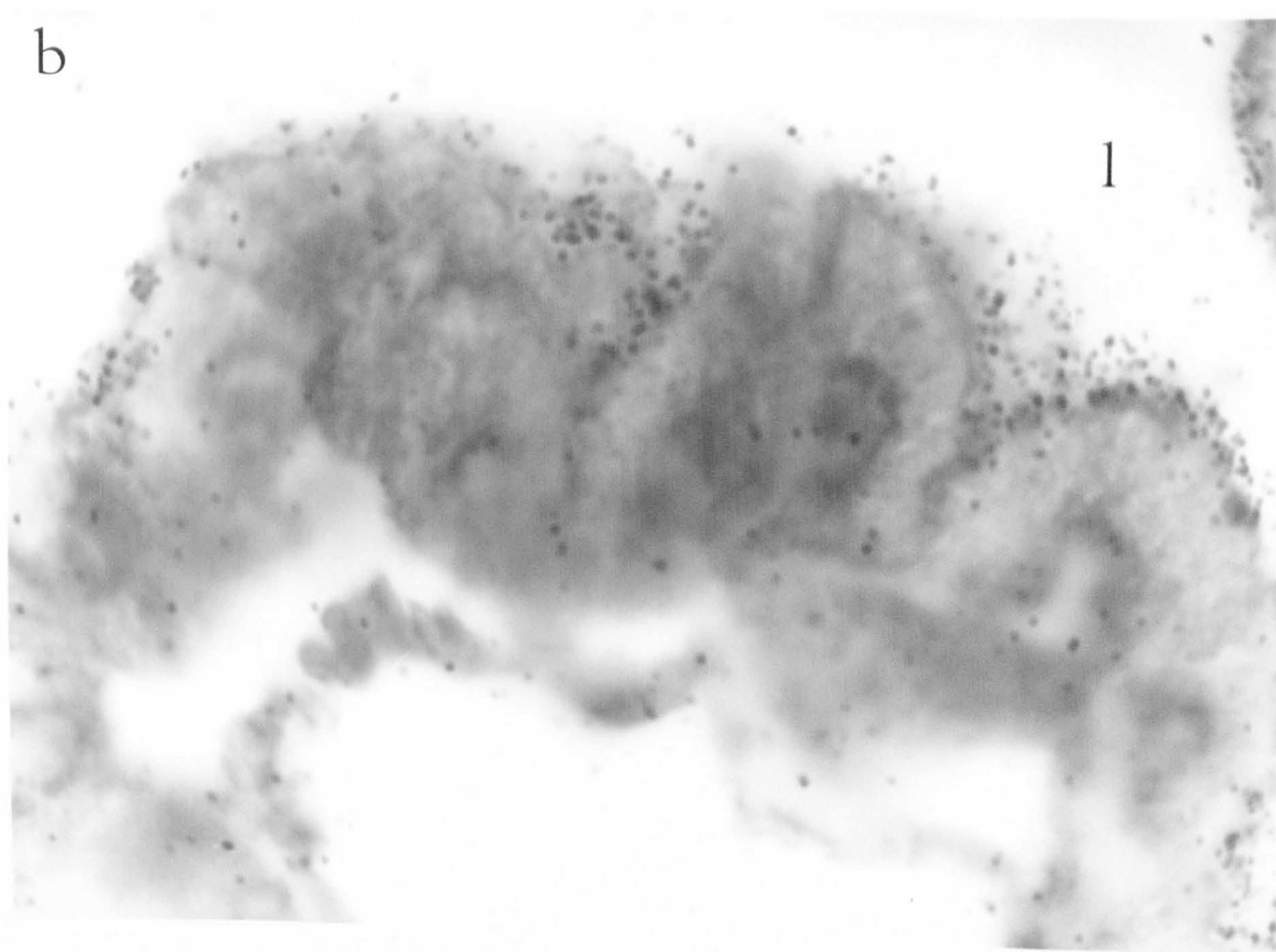
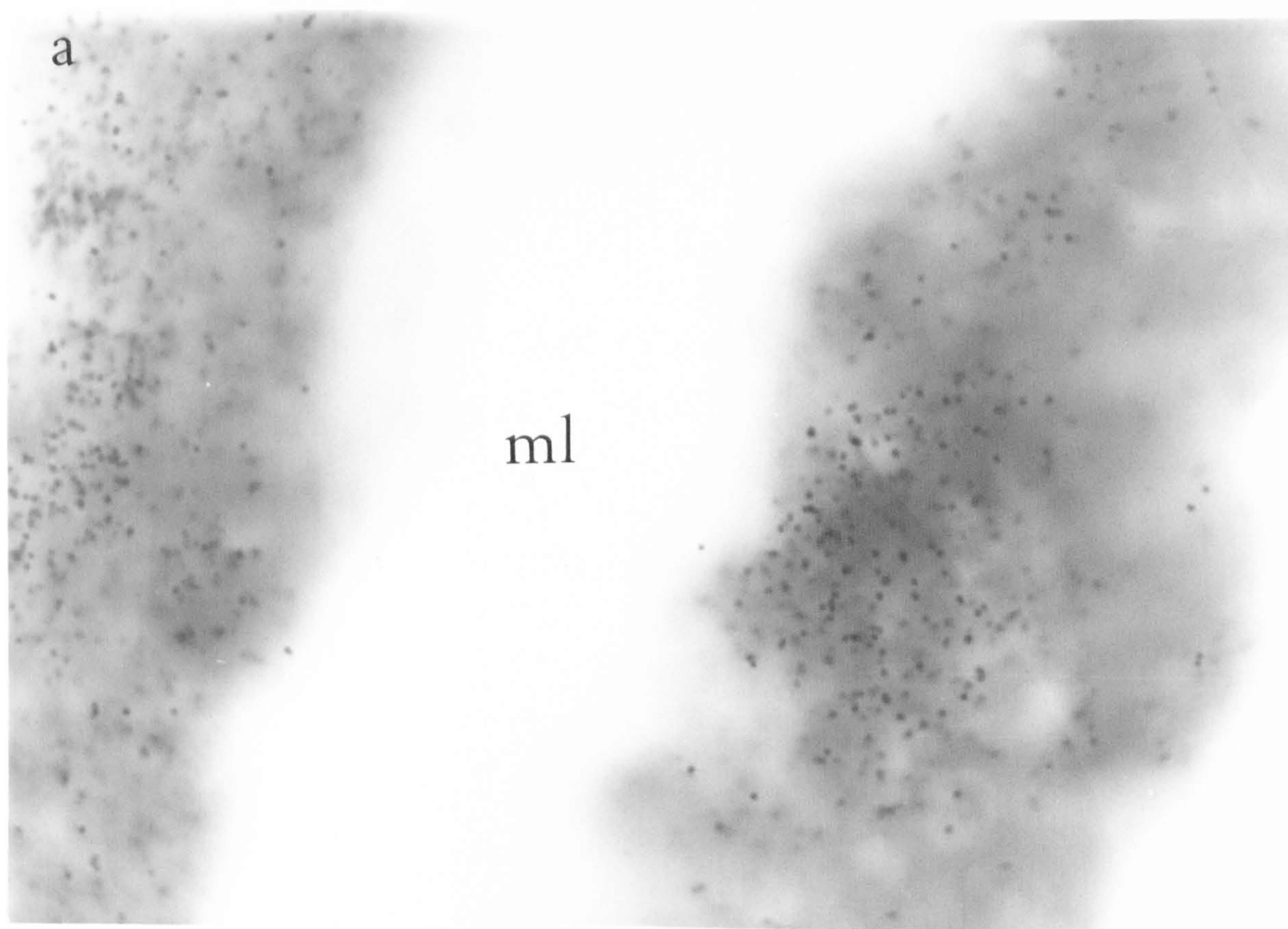


PLATE 16

(a, b) Incorporation of tryptophan-[H₃] into thoracic midgut of Aedes togoi. The isotope was injected at 91.5 hours pbm. On the left (a) of the photograph the focus is on the tissue. ap = apical leaflets. bm = basement membrane. Note the absence of label over the apical leaflets.

(c) Incorporation of valine-[H₃] into abdominal midgut of Anopheles labranchiae atroparvus. The isotope was injected at 90.5 hours pbm. ml = midgut lumen. Note that the label also appears over the apical leaflets.

a



b



c

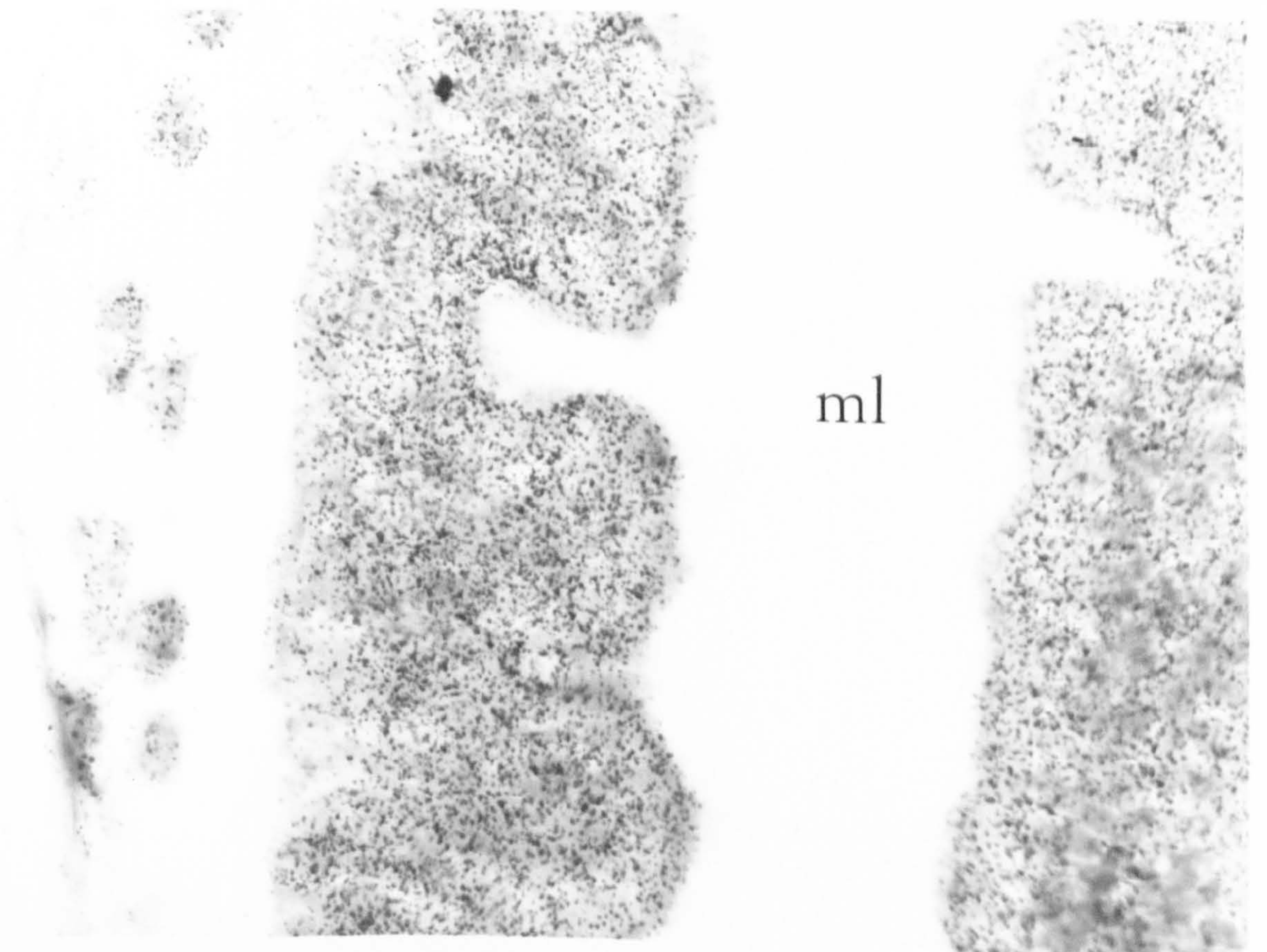


PLATE 17

Incorporation of tryptophan-[H₃] into ileum of Aedes togoi.
The isotope was injected at 91.5 hours pbm. l = lumen of
ileum. c = cuticular intima.

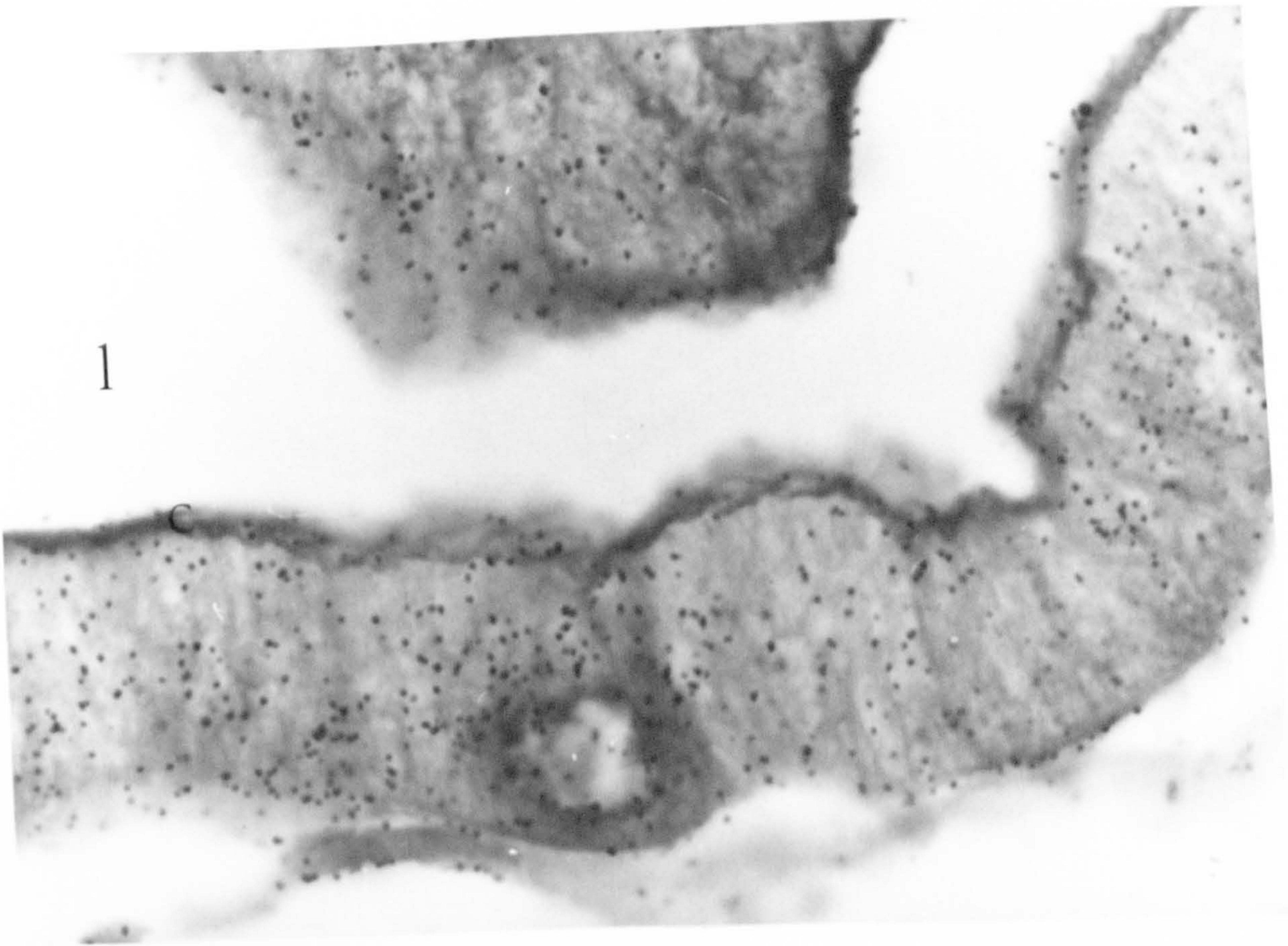
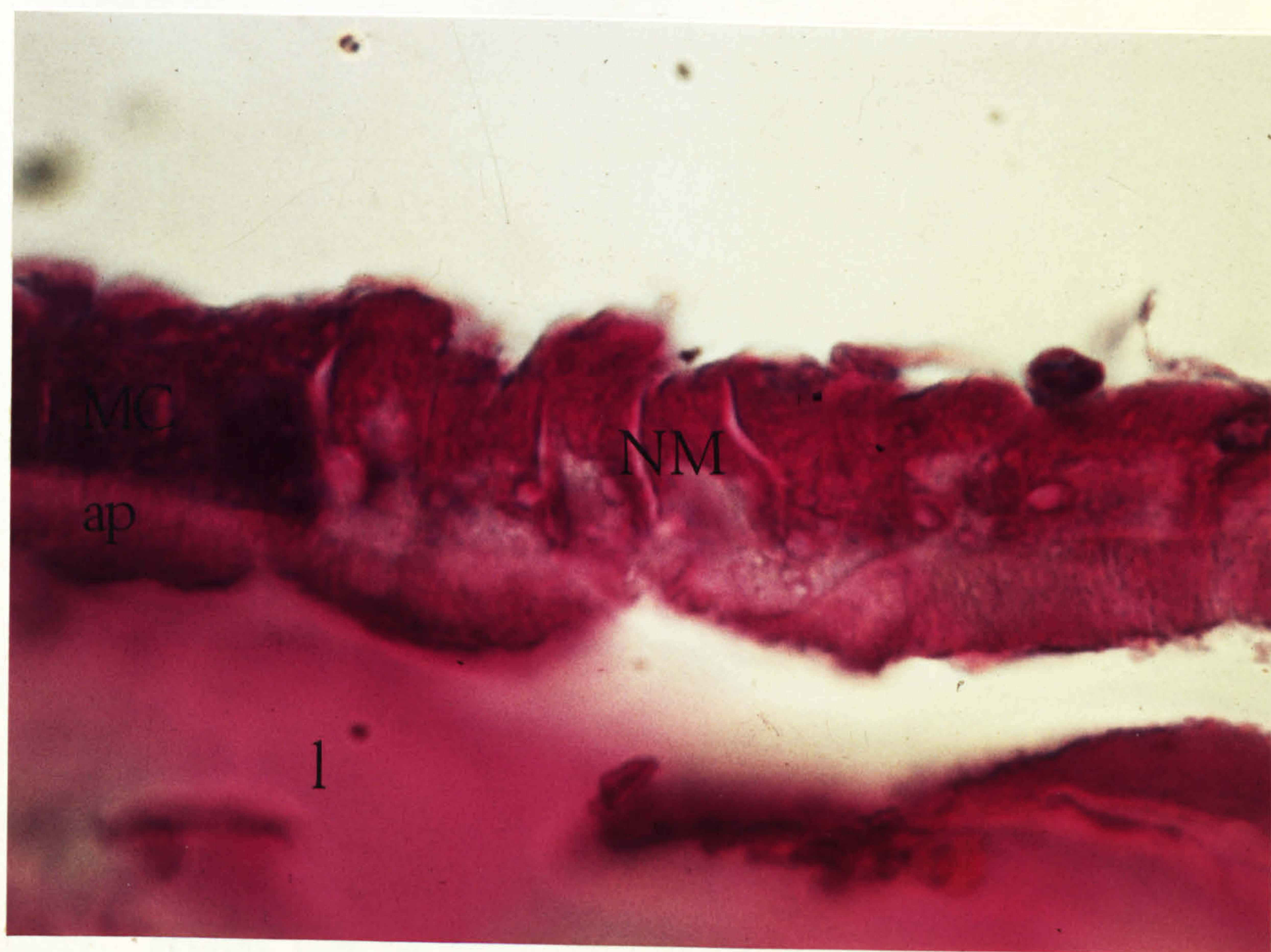


PLATE 18

Focal necrosis in abdominal midgut of Anopheles labranchiae atroparvus caused by migrating microfilariae of Brugia patei. This individual was fixed in FA at 2 hours pbm and stained with CHP. ap = apical leaflets. l = midgut lumen. MC = normal midgut cells. NM = necrotic midgut cells exhibiting characteristic intense acidophilia with cytoplasmic vacuolation and loss of nuclear detail.



AUTORADIOGRAPHIC AND HISTOCHEMICAL OBSERVATIONS ON THE OVARIES AND
ASSOCIATED TISSUES OF AEDES TOGOI AND ANOPHELES LABRANCHIAE ATROPARVUS

INTRODUCTION

The histological structure of the mosquito reproductive tract has been reviewed by Christophers (1960), and by Giglioli (1964). The physiological basis of ovary development has been comprehensively reviewed by Laurence (1977). The following introductory account has drawn heavily on these sources.

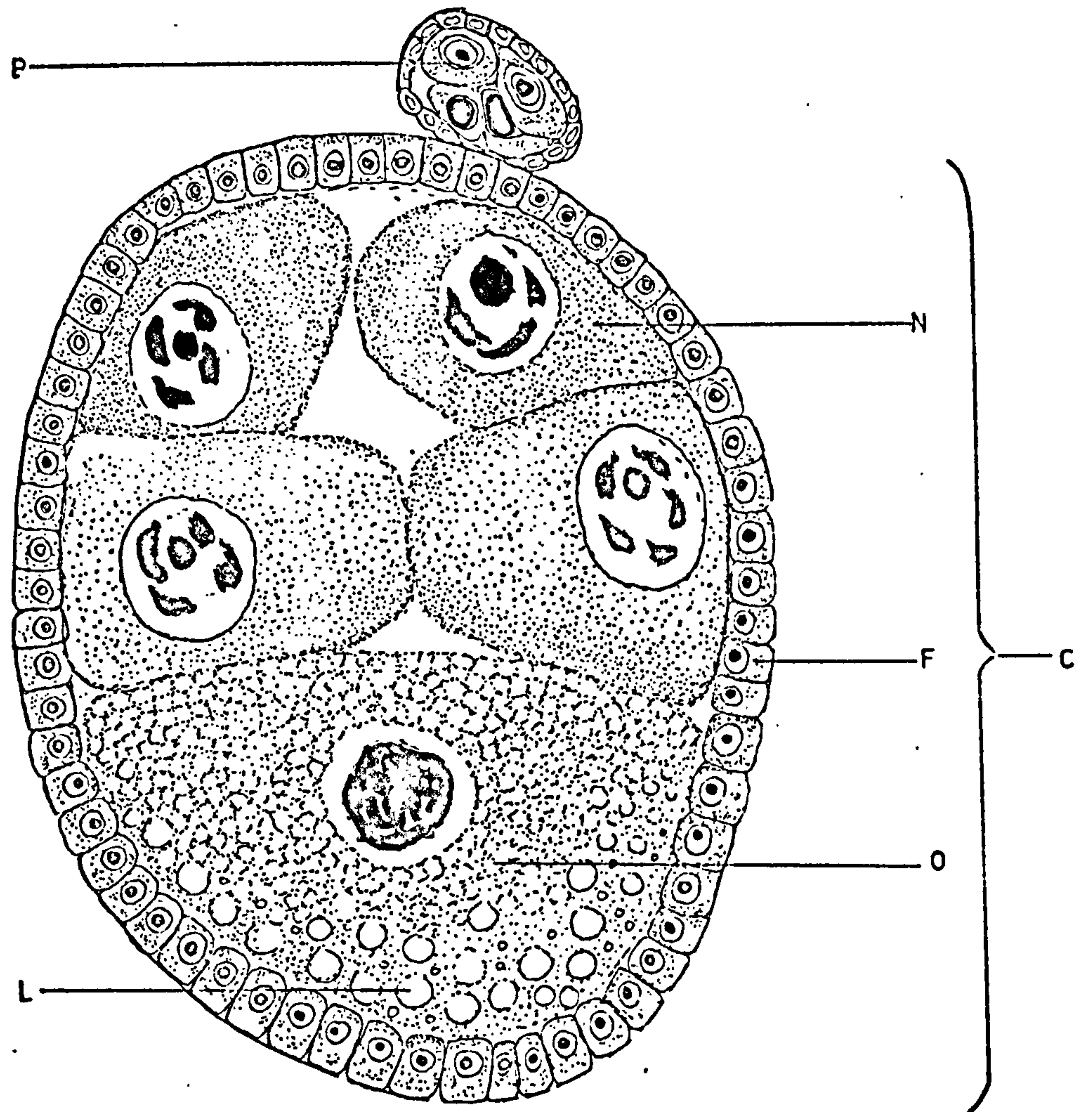
The mosquito ovaries are paired structures situated on either side of the abdominal cavity, and extending from segments V-VII. From its opening in the genital atrium the common oviduct proceeds anteriorly dividing into two lateral oviducts which enter into each ovary. The extension of a lateral oviduct into an ovary forms a central chamber known as the calyx. Arising from the calyx are radially arranged follicular tubes (ovarioles).

As in other Diptera, the mosquito ovary is of the polytrophic meroistic type, each ovariole containing several distinct egg follicles, with the current (primary) follicle in each ovariole, clearly differentiated into an oocyte, 7 nurse (nutritive) cells; the whole follicle being enclosed by a monolayer of cuboidal epithelium (Fig 6). The penultimate and third follicles are poorly differentiated by comparison. The first two or three follicles within each ovariole, constitute the vitellarium, and the current follicle is situated nearest the calyx. Posterior to the vitellarium is a cap of cells known as the germarium from which new follicles proliferate.

The mosquito ovary is well supplied by trachea, and by a network of muscle and cellular membranes, details of which are given by Giglioli (1964).

Whilst sugar feeding is necessary for the prolonged survival of the female mosquito (Nayer and Sauerman, 1975a), blood feeding is essential for egg

Fig 6 Histological features of current follicle of Anopheles labranchiae atroparvus at the resting stage



c = current follicle, p = penultimate follicle, f = follicular epithelium,
n = nurse cells, o = oocyte, L = lipid yolk

development. This latter remark needs qualifying by stating that whilst most mosquitoes require a meal of blood in order to mature a batch of eggs (anautogeny), a lesser number can mature the first batch of eggs without recourse to blood feeding (autogeny). Aedes togoi is autogenous depending upon the larval reserves that are carried over onto the pupal stage (Laurence, 1964). Autogeny in mosquitoes has been reviewed by Spielman (1971).

Christophers (1911) divided a follicular cycle into 5 stages (Fig 7). The first two are pre-vitellogenic. Stage I is the relatively undifferentiated state of the follicle after emergence from the pupa. Growth from Stage I to Stage II is accomplished by mitotic activity of the follicular epithelium (Laurence and Simpson, 1974). Stage II is complete when a small amount of lipid inclusions can be seen as a crescentic rim of refractile material at the base of the oocyte. This marks the end of the pre-vitellogenic phase. From the work of Lea (1963; 1967) and more recently Gwadz and Spielman (1973), current thinking suggests that the pre-vitellogenic phase of ovarian development is controlled by the corpora allata and/or juvenile hormone.

Stage II-III, the deposition of protein - carbohydrate yolk (Laurence and Roshdy, 1963; Hagadorn and Judson, 1972; Troy et al, 1975) will not begin in an anautogenous female without the trigger provided by a blood meal. Ovary development after the blood meal is dependent and regulated by the release of egg development neurosecretary hormone (EDNH), assumed to be synthesised in the medial neurosecretary cells (MNC) perikarya in the brain, and known to be stored in a neurohaemal organ the corpus cardiacum (Lea, 1972; Meola and Lea, 1972a). Hormone is then metered into the haemolymph from the corpus cardiacum by a stimulus arising from the blood meal.

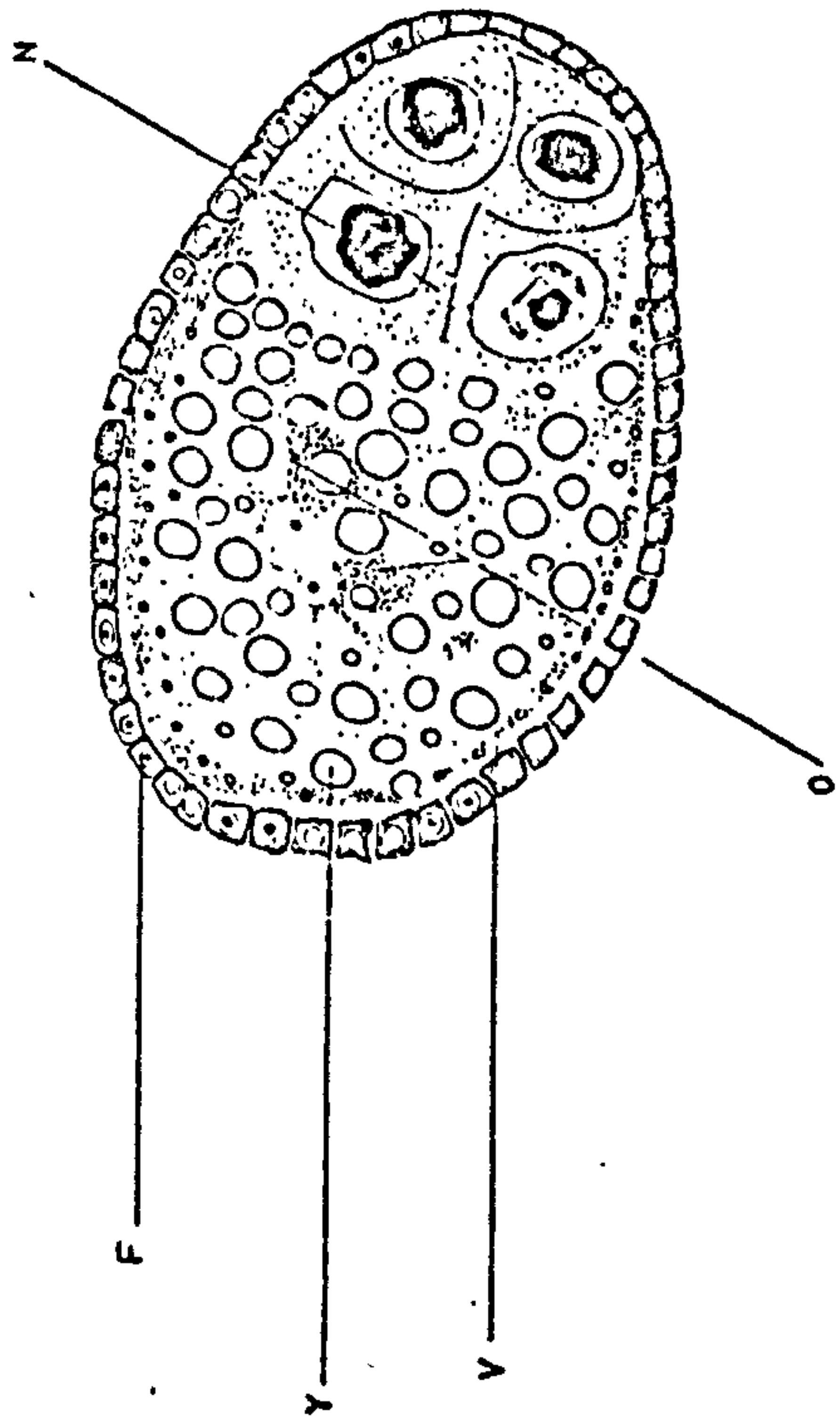
Spielman, Gwadz and Anderson (1971) have demonstrated that exogenous ecdysone initiates ovarian development in non blood females. Schlaeger, Fuchs and Kang (1974) suggested that the blood meal triggers the synthesis, activation or release of endogenous ecdysone.

During Stage II-III the surface of the oocyte, the oolemma is elaborated into a dense array of microvilli, and actively proliferates numerous

Fig 7 - Histological changes in current follicle of Anopheles labranchiae atroparvus after a blood meal.

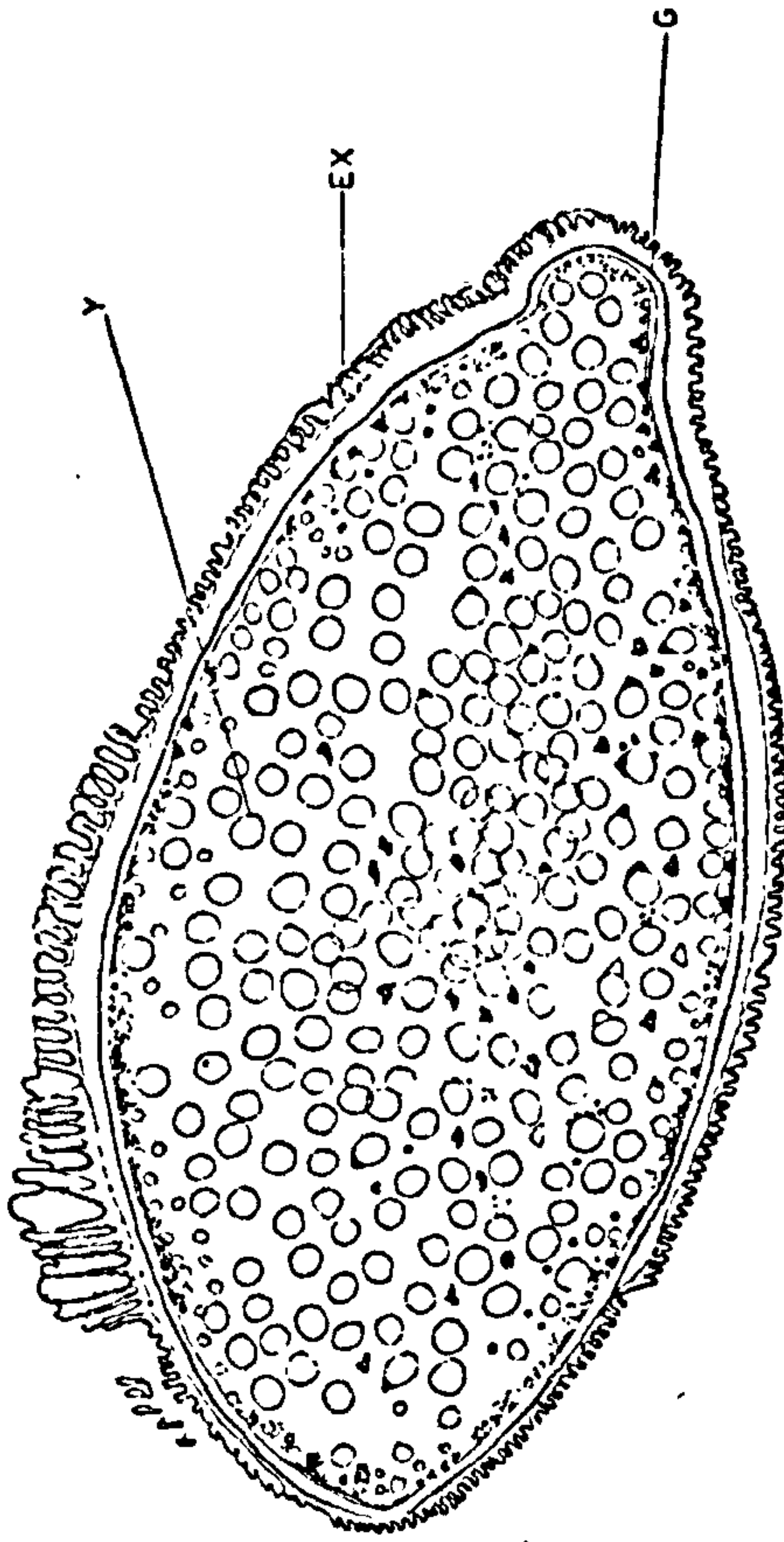
Stage II-III

(a) 24 hours pbm

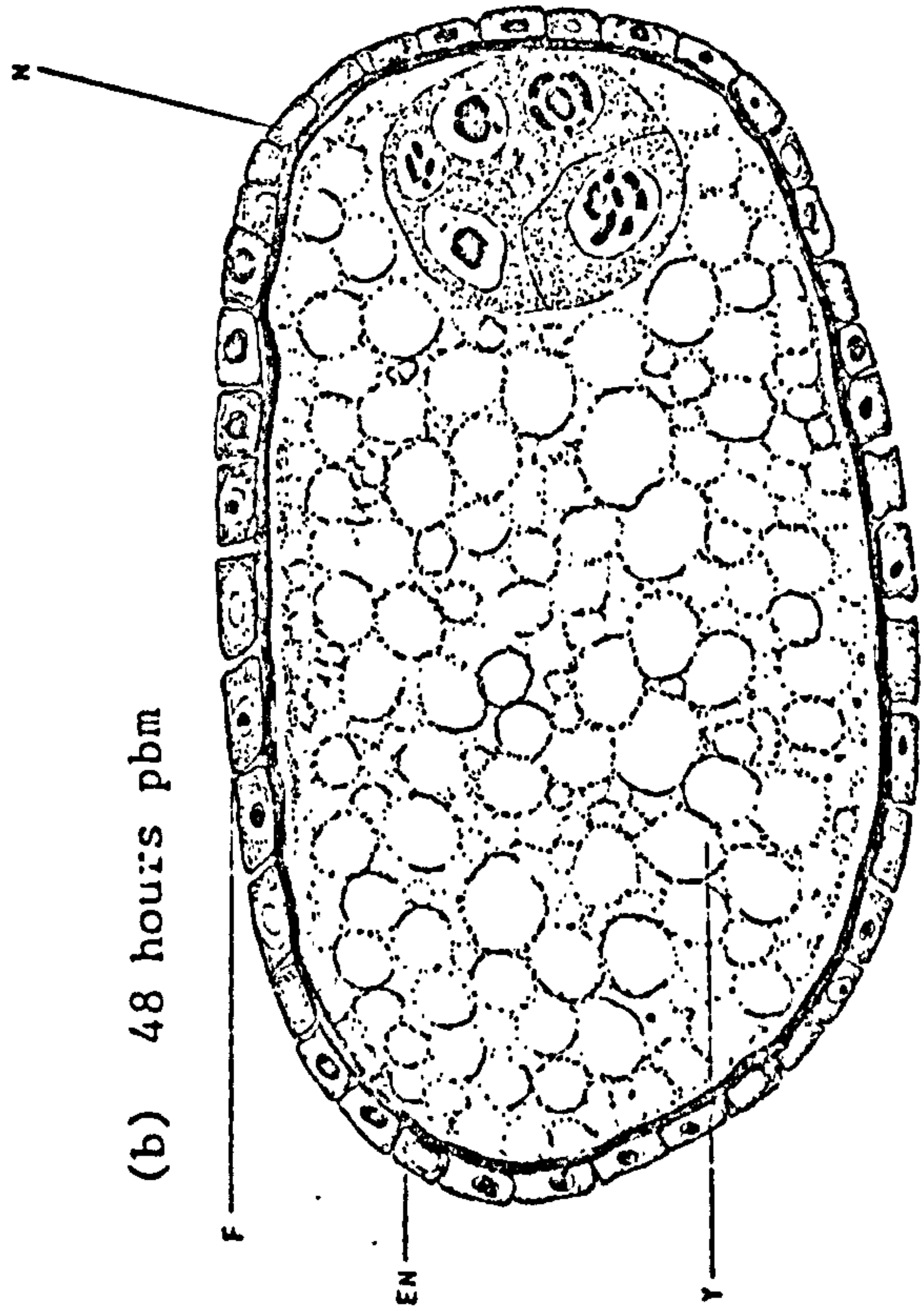


Stage IV V

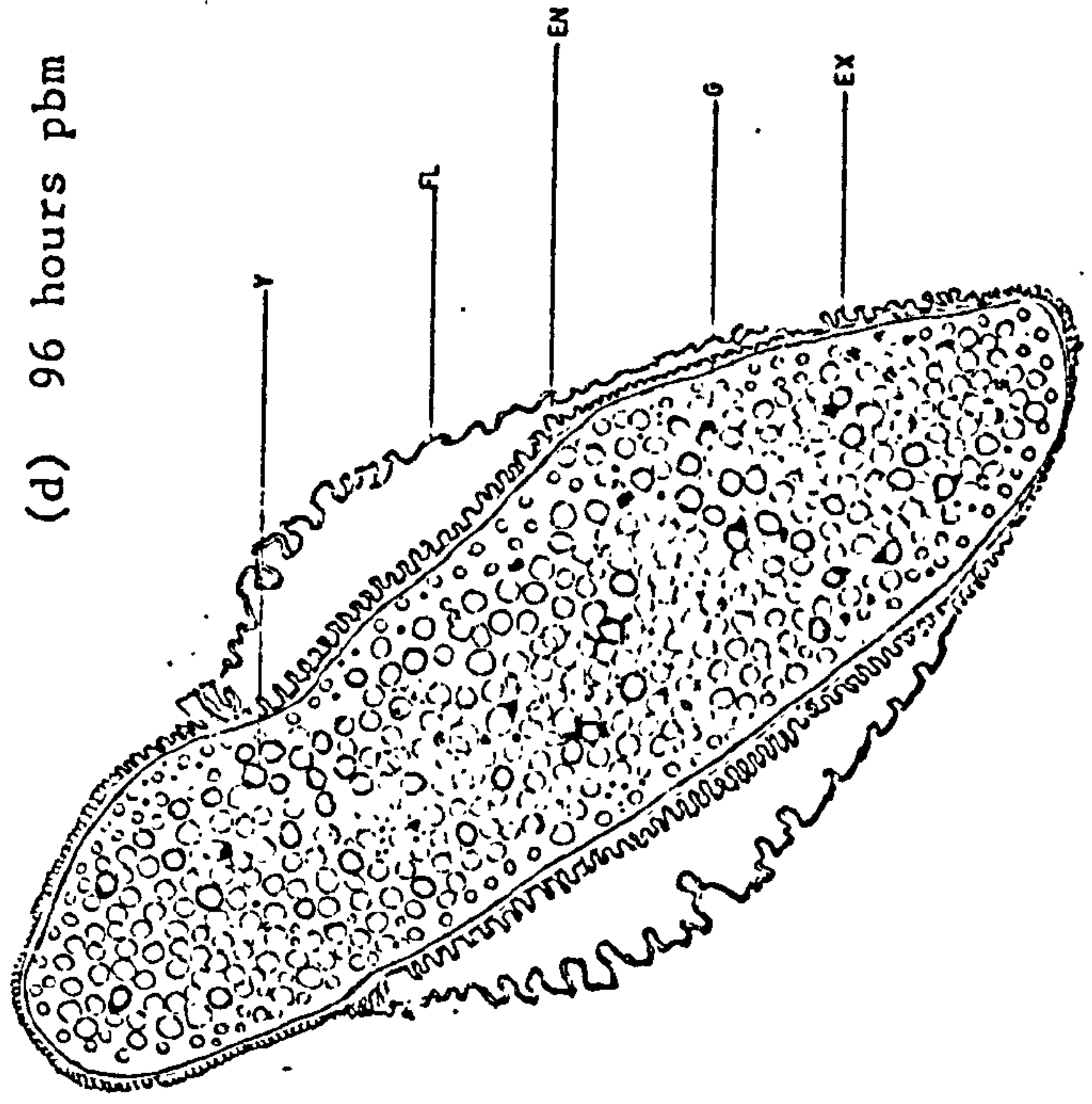
(c) 72 hours pbm



(b) 48 hours pbm



(d) 96 hours pbm



F = follicular epithelium, N = nurse cells, O = oocyte,
Y = yolk spheres, V = vitelline membrane, EN = endochorion,
EX = exochorion, FL = floats, G = glycogen.

pinocytotic vesicles (Roth and Porter, 1964). During this micro-pinocytotic phase exogenous yolk precursors are incorporated into the ooplasm (Anderson and Spielman, 1971). The source of the extra ovarian yolk protein (vitellogenin) is the fat body, and this organ will synthesise vitellogenin without the stimulus of a blood meal, providing an injection of β -ecdysone is given (Fallon et al, 1973).

The yolk accumulates in the oocyte until the nucleus is obscured (Stage III). The nurse cell nuclei are extruded through a pore called the micropyle, and the egg is steadily elongating (Stage IV). Development of the egg is complete when the egg membranes are fully formed and sculptured (Stage V). Vitelline membrane formation is recognisable at 24 hours pbm, and by 48 hours pbm, the formation of the vitelline membrane and endochorion is completed (Mathew and Rai, 1975).

In this study, autoradiographic observations on the ovary and associated tissues of Aedes togoi and Anopheles labranchiae atroparvus are described, including some preliminary histochemical data, and the results discussed in relation to the physiology of follicular development.

RESULTS

1. Incorporation into the current follicle

(a) Increase in length

The rate of elongation of the current follicle of Aedes togoi and Anopheles labranchiae atroparvus after a meal of blood is illustrated in Fig 8. In both species the follicles increased steadily until approximately 72 hours after engorgment.

(b) Uptake of nucleosides

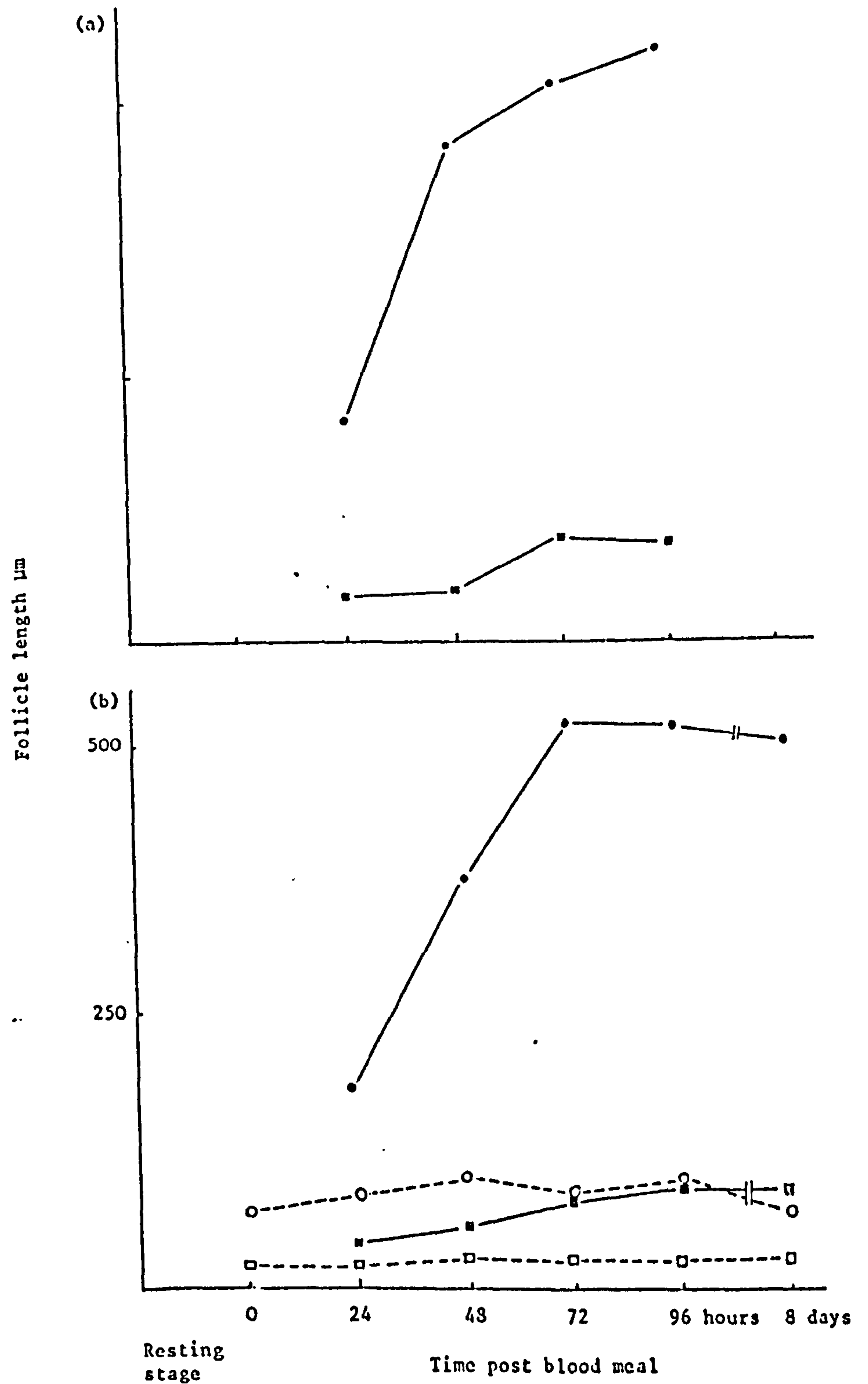
The incorporation of thymidine-[H₃] into the current follicle of autogenous Aedes togoi (Plate 19a) has already been described by Laurence and Simpson, (1974). These workers showed that the follicular epithelium of follicles at Stage II to early Stage III showed intense incorporation of thymidine-[H₃] into the nuclei of the follicular epithelium. There was progressively less intense incorporation during Stage III when eventually the follicular epithelium ceased to show mitotic activity (Laurence and Simpson, 1974).

The resting stage follicles of Aedes togoi incorporated uridine-[H₃] (Plate 19b) and in individuals injected at 26.5 hours pbm the follicular epithelium and nurse cells were equally labelled with uridine-[H₃]. The degree of uridine-[H₃] label over the oocyte was lower. In individuals injected with uridine-[H₃] at 45 hours pbm, incorporation into the follicular epithelium had significantly decreased. The nurse cells had been extruded. The degree of uridine-[H₃] label over the oocyte was still low.

(c) Uptake of carbohydrates

In Aedes togoi and Anopheles labranchiae atroparvus, there was negligible incorporation of glucose-[H₃], galactose-[H₃] and glucosamine-[H₃] in individuals that had received an injection of one of these isotopes at 4 days pbm. In earlier follicles the situation was different. In Anopheles labranchiae atroparvus injected at 24 hours pbm with glucose-[H₃], the follicular epithelium and nurse cells were uniformly

Fig 8 Increase in follicle length of (a) *Aedes togoi*
(b) *Anopheles labranchiae atroparvus* after a blood meal



current follicle -●- : penultimate follicle -■-:
current follicle of unfed control -○- : penultimate follicle of unfed control -□-

labelled. The label over the oocyte was lower. The follicular epithelium still incorporated glucose-[H₃] in individuals injected at 48 hours pbm. However, the egg membranes of individuals injected at 72 or 96 hours pbm showed negligible amounts of the isotope. The nurse cells were able to incorporate glucose-[H₃] until shortly before extrusion. The oocyte showed an ability to incorporate glucose-[H₃] throughout the period 24-96 hours pbm. The label was low at 24 hours pbm but higher and uniform at 48-96 hours pbm. The results of the glucose-[H₃] injections are summarised in Table 25.

Very little galactose-[H₃] became incorporated into the current follicle of Anopheles labranchiae atroparvus within the period 24-96 hours pbm.

(d) Uptake of amino acids

In Aedes togoi and Anopheles labranchiae atroparvus there was relatively little incorporation of labelled, non-phenolic amino acids in the current follicles of individuals that had been injected at 4-5 days pbm.

In Anopheles labranchiae atroparvus that had been injected with either phenylalanine-[H₃], tyrosine-[H₃] or DOPA-[H₃] within a time range of 24-96 hours pbm, the incorporation into the follicular epithelium tended to be higher than the other follicular components. In individuals that had been injected at 24 hours pbm with either phenylalanine-[H₃] or tyrosine-[H₃], the follicular grain density was more intense at the interface between the follicular epithelium and the yolk spheres. The nurse cells incorporated labelled phenolic amino acid until shortly before extrusion. The oocyte continued to show some degree of incorporation throughout the period 24-96 hours pbm. These results are summarised in Table 26.

2. Incorporation into the second (penultimate) follicle

(a) Increase in length

The rate of elongation of the second follicle of Aedes togoi and Anopheles labranchiae atroparvus after a meal is illustrated in Fig 8.

Table 25 Incorporation of glucose-H3 into current follicle of Anopheles
labranchiae atroparvus

Time of injection (hours pbm)	No mosquitoes used	Mean grain density $\bar{x} \pm$ standard error of mean			
		Follicular epithelium	Nurse cells	Oocyte/yolk	Background
24	3	14.27 \pm 1.33	16.80 \pm 0.90	7.80 \pm 0.72	1.96 \pm 0.18
48	3	23.87 \pm 2.58	21.67 \pm 2.60	19.8 \pm 5.48	2.64 \pm 0.15
72	2	4.00 \pm 0.70	Extruded	14.30 \pm 4.58	1.60 \pm 0.14
96	3	3.80 \pm 0.49	Extruded	18.80 \pm 4.35	3.09 \pm 1.19

Each animal was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x})
per unit area of follicular tissue = $\frac{\text{(total number grains counted)}}{\text{number mosquitoes used} \times 5 \text{ unit areas per tissue}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{\text{(total number grains counted)}}{\text{number mosquitoes used} \times 100 \text{ unit areas}}$

1 unit area = 64 μm^2 .

In both species the follicles increased slightly in length until 72 hours pbm. No further increases in length were noted at 96 hours pbm.

(b) Uptake of nucleosides

The incorporation of thymidine-[H₃] into the second follicle merits an analytical treatment similar to that given by Laurence and Simpson (1974) in their study of the changes in the follicular epithelium of anautogenous and autogenous mosquitoes in relation to blood feeding.

In this study time was not available to collect data quantitative on thymidine-[H₃] incorporation into the follicles in relation to follicle differentiation. It is noted here that incorporation of thymidine-[H₃] took place in the second follicle of Aedes togoi between 3-24 hours pbm.

In Aedes togoi that had received an injection of uridine-[H₃], incorporation in the second follicles was low at 26.5 hours pbm. In individuals injected at 45 hours or at 5, 6 or 8 days pbm, the level of incorporation of uridine-[H₃] into the second follicle was higher, with all the follicular elements showing label. In Anopheles labranchiae atroparvus, individuals that had been injected with uridine-[H₃] at 45 hours pbm, also incorporated the isotope into the second follicle. In both mosquito species the distribution of uridine-[H₃] within the second follicle, appeared to be random.

(c) Uptake of carbohydrates

In Aedes togoi and Anopheles labranchiae atroparvus there was some incorporation of glucose-[H₃], but negligible incorporation of galactose-[H₃] and glucosamine-[H₃] into the second follicle of individuals injected at 4 days pbm.

In Anopheles labranchiae atroparvus the relative level of incorporation of glucose-[H₃] by the second follicle was similar in individuals that had received the injection of isotope at either 24, 48 or 72 hours pbm. The label over the second follicle of individuals that had been injected at 96 hours pbm was slightly lower (Table 27). Within the second follicle, the radioactivity appeared to be randomly distributed.

Table 26 Incorporation of phenylalanine-[113], tyrosine-[113] and DOPA-[113] into the current egg follicle of Anopheles labranchiae atroparvus

Isotope	Time of injection (hours pbm)	No mosquitoes used	Mean grain density $\bar{x} \pm$ standard error of mean			
			Follicular epithelium	Nurse cells	Oocyte/yolk	Background
Phenylalanine	24	1	76.40 \pm 4.56	53.60 \pm 4.53	28.00 \pm 3.40	<2
	48	1	58.00 \pm 5.15	25.60 \pm 1.20	16.40 \pm 0.45	3.33 \pm 0.29
	72	1	Unlabelled	Extruded	5.00 \pm 0.90	3.11 \pm 0.19
	96	1	Unlabelled	Extruded	Unlabelled	<2
Tyrosine	24	3	75.60 \pm 7.30	59.50 \pm 7.46	13.20 \pm 3.05	1.72 \pm 0.27
	48	2	44.10 \pm 4.96	43.20 \pm 2.90	10.20 \pm 2.02	1.10 \pm 0.15
	72	3	Unlabelled	Extruded	11.67 \pm 2.29	2.83 \pm 0.87
	96	1	Unlabelled	Extruded	5.80 \pm 0.76	<1
DOPA	24	2	37.20 \pm 8.67	5.0 \pm 1.40	8.80 \pm .08	1.17 \pm 0.17
	48	3	9.20 \pm 1.76	4.0 \pm .98	14.80 \pm 1.55	1.09 \pm 0.16
	72	3	10.33 \pm 2.14	Extruded	Unlabelled	1.03 \pm 0.10
	96	3	8.87 \pm 1.68	Extruded	Unlabelled	<1

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x})
per unit area of follicular tissue = $\frac{\text{(total number grains counted)}}{\text{number mosquitoes used} \times 5 \text{ unit areas/tissue}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{\text{(total number grains counted)}}{\text{number mosquitoes used} \times 100 \text{ unit areas}}$

1 unit area = 64 μm^2 .

(d) Uptake of amino acids

The second follicle of Aedes togoi and Anopheles labranchiae atroparvus incorporated all of the labelled amino acids that were used for this study, except for proline-[H₃]. In Anopheles labranchiae atroparvus which had received an injection of either phenylalanine-[H₃], tyrosine-[H₃] or DOPA-[H₃] within the period 24-96 hours pbm, incorporation into the second follicle was variable. With phenylalanine-[H₃], incorporation was more intense at 72-96 hours pbm. With tyrosine-[H₃] there was no association between incorporation in the second follicle and the time of injection of the isotope. DOPA-[H₃] incorporation into the second follicle was uniform (Table 28). Within each follicle irrespective of the phenolic isotope used, the label between the various follicular elements was comparable.

It is concluded that within the period 24-96 hours pbm the follicular epithelium of the current follicle showed maximum incorporation at 24-48 hours pbm, the nurse cells are active until shortly after extrusion, and the oocyte can incorporate precursors even after the vitellogenic phase. In comparison, incorporation into the second follicle was none differential (Plate 20) and continued throughout the period 24-96 hours pbm.

3. Incorporation into the oviducts

In Aedes togoi, thymidine-[H₃] labelling of the calyx cells occurred in individuals that had been injected between 3-24 hours pbm, but there was no recognisable pattern in the degree of uridine-[H₃] labelling of either the calyx cells or the epithelial cells of the oviducts.

In both Aedes togoi and Anopheles labranchiae atroparvus, glucosamine-[H₃] was incorporated into the oviduct epithelium (Table 29) and genital atrium. This label was in marked contrast to the negligible incorporation of glucosamine-[H₃] into other parts of the female reproductive tract. Galactose-[H₃] incorporation into the oviduct epithelium was also negligible in both species. Glucose-[H₃] was incorporated into the oviduct epithelium of Anopheles labranchiae atroparvus at 24-72 hours pbm (Table 29).

Table 27 Incorporation of glucose-[H₃] into penultimate follicle of *Anopheles labranchiae atroparvus*

Time of injection (hours pbm)	No mosquitoes used	Mean grain density \bar{x} ± standard error of mean	
		Follicle	Background
24	3	13.13±1.84	1.96±0.18
48	3	13.00±2.42	2.64±0.15
72	2	20.60±2.64	1.60±0.14
96	3	8.13±0.78	3.09±1.19

Each animal was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x})
per unit area of follicular tissue = $\frac{(\text{total number grains counted})}{\text{number mosquitoes used} \times 5 \text{ unit areas per tissue}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{(\text{total number grains counted})}{\text{number mosquitoes used} \times 100 \text{ unit areas}}$

1 unit area = 64 μm^2 .

The oviduct epithelium also incorporated all of the labelled amino acids that were employed in this study except for proline-[H₃].

In Anopheles labranchiae atroparvus there was no relationship between incorporation of [H₃]-phenolic amino acid and the time of injection, at least within the period 24-96 hours pbm (Table 30).

4. Histochemical observations

These preliminary observations were restricted mainly to the period which had also been studied by autoradiography, ie 24-96 hours pbm. Unless stated otherwise the staining results reported here apply equally to both Aedes togoi and Anopheles labranchiae atroparvus.

(a) Current follicle

Nucleic acids. The cytoplasm in the follicular epithelium of the current follicles of Aedes togoi and Anopheles labranchiae atroparvus stained intensely with pyronin at 24-48 hours pbm (Plate 21). Pyronin staining was also intense in the cytoplasm of the nurse cells at 24 hours pbm (Plate 21a) but at 45-48 hours the staining was reduced and particulate in appearance (Plate 21b). The cytoplasm of the oocyte was only moderately stained with pyronin during the period 24, 48, 72 and 96 hours pbm, but the yolk spheres were unstained (Plate 21). Pyronin staining in the current follicle was prevented by prior incubation of the sections in ribonuclease indicating that the staining was due to RNA.

Carbohydrates. Since all PAS staining in the follicles was prevented by prior acetylation with acetic anhydride in pyridine, and staining of the acetylated sections restored by saponification with ethanolic KOH, it is concluded that PAS staining in the follicles of paraplast-embedded mosquito sections was due to carbohydrate.

Periodate oxidation was necessary to induce staining with Schiff's reagent of the carbohydrate component of the follicles. The use of an aldehyde blocking agent (sodium borohydride, dimedone etc) interposed between oxidation and Schiff's reagent prevented staining from taking

Table 28 Incorporation of phenylalanine-[H₃], tyrosine-[H₃]
and DOPA-[H₃] into the penultimate follicle of
Anopheles labranchiae atroparvus

Isotope	Time of injection (hours pbm)	No mosquitoes used	Mean grain density \bar{x} ± standard error, of mean
Phenylalanine	24	1	59.60±3.97
	48	1	51.20±1.00
	72	1	>4100
	96	1	93.0 ±3.34
Tyrosine	24	2	59.60±5.92
	48	2	41.20±4.34
	72	2	75.30±3.54
	96	1	25.40±2.37
DOPA	24	2	6.00±0.77
	48	3	4.47±0.97
	72	3	6.07±0.57
	96	3	6.13±0.87

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x})
per unit area of follicular tissue = $\frac{(\text{total number grains counted})}{\text{number mosquitoes used} \times 5 \text{ unit areas/tissue}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{(\text{total number grains counted})}{\text{number mosquitoes used} \times 100 \text{ unit areas}}$

1 unit area = 64 μm^2 .

The background mean for each amino acid was <3 g/unit area.

place. It was concluded that dialdehyde groups present on 1:2 glycols were responsible for PAS staining of the follicles. That proportion of the 1:2 glycol-rich material that was removable by amylase treatment was considered to be glycogen.

Glycogen, as detected by Best's carmine, PAS and sodium bisulphite toluidine blue staining, appeared to be absent from 24 and 48 hour follicles. The distribution of glycogen within 72-96 hour follicles was mainly restricted to the cytoplasm of the oocyte between the yolk spheres either as tiny granules or as larger masses capping individual spheres (Plate 22). In ripe eggs glycogen was concentrated more in the periplasm than in the deeper regions of the egg.

The absence of detectable glycogen in the current follicle at 24 hours pbm ties in with the low degree of incorporation of glucose- $[H_3]$ into the follicle at this time.

The yolk spheres also contained 1:2 glycol rich material that was resistant to removal by amylase (Plate 23a). No 1:2 glycol-rich material was detected in the nurse cells, follicular epithelium and egg membranes.

Polyanions, detectable with AB (2.5), LID or HID methods, appeared to be absent from the current follicles. Although the CEC method indicated that at dye bath concentrations of 0.4-0.6 M $MgCl_2$ staining in the ooplasm around the yolk spheres was above background, it is considered that this result alone is insufficient to prove the presence of acid mucosubstance in the follicles.

It is interesting to record that in sections oxidised by periodate and followed by LID or HID reagents, purple staining was present in the follicles with a distribution matching that of glycogen.

Proteins. Proteins were present in the yolk spheres. The diazotization-coupling reaction and bromphenol blue stained the spheres intensely (Plate 23b) whereas only moderate staining was seen with the DMAB-nitrite method. Barnett and Seligmans' technique for proteins rich in side chain carboxyls did not show any staining of the follicles.

Table 29 Incorporation of glucosamine-[H₃], galactose-[H₃] and glucose-[H₃] into the oviducts of Aedes togoi and Anopheles labranchiae atroparvus

Isotope	Time of injection (hours pbm)	No mosquitoes used	Mean grain density \bar{x} ± standard error of mean	
			Oviducts	Background
(a) <u>Aedes togoi</u>				
Glucosamine	95	1	19.00±2.60	0.86±0.09
Galactose	92.5	1	2.60±0.24	1.03±0.10
(b) <u>Anopheles labranchiae atroparvus</u>				
Glucosamine	95	1	23.20±2.01	1.74±0.16
Galactose	92.5	1	5.80±1.91	1.03±0.09
Glucose	24	2	13.40±0.59	1.96±0.18
	48	3	14.67±3.14	2.64±0.15
	72	1	12.60±1.04	1.50±0.13
	96	1	4.80±0.56	1.80±0.13

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x})
per unit area of follicular tissue = $\frac{\text{(total number grains counted)}}{\text{number mosquitoes used per tissue x 5 unit areas}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{\text{(total number grains counted)}}{\text{number mosquitoes used x 100 unit areas}}$

1 unit area 64 μm^2 .

The nurse cells also contained much basic protein in comparison to the ooplasm and follicular epithelium. The egg membranes of ripe eggs were mostly unreactive to protein stains but the endochorion in 48-72 hour eggs reacted strongly to bromphenol blue and the diazotization coupling reaction.

Lipids. The yolk spheres also stained positively with the Oil red O method for neutral fat. This staining was prevented if the cryostat sections were immersed in a fat solvent such as xylene or chloroform prior to staining with Oil red O. The vitelline membrane also was reactive to Oil red O, but no fat was demonstrated in the nurse cells and follicular epithelium.

Enzymes. In the ripe follicle β -glucosaminidase activity was localised as tiny, bright red granules interspersed between the yolk spheres, whereas non-specific esterase activity was present as blue-purple crescentic caps of material on each of the yolk spheres. These enzymes did not appear to be present elsewhere in the follicle.

(b) Second follicle

The cytoplasm of the penultimate follicles stained moderately with pyronin at 24 hours pbm but more strongly at 48-96 hours pbm (Plate 21b). The pyronin staining of the follicles was prevented by a prior incubation of the sections in ribonuclease indicating that pyronin basophilia was due to the presence of RNA. No carbohydrate was demonstrated in the follicles but basic proteins were present in the nurse cells. The follicles contained traces of β -glucosaminidase and non specific esterase activity but no acid phosphatase. The follicles were unreactive to Oil red O indicating the absence of neutral lipids.

(c) Oviducts

The cytoplasm of the oviduct epithelium exhibited a weak to moderate ribonuclease labile pyronin basophilia. Trace amounts of glycogen was present in the cytoplasm of the oviduct epithelium but mucosubstances were absent. However, the PAS and Alcian blue techniques indicated that the cuticular intima of the oviducts contained 1:2 glycol groups and polyanionic material. The cuticular intima also contained -SS- groups

Table 30 Incorporation of phenylalanine-[H₃], tyrosine-[H₃] and DOPA-[H₃] into the oviducts of *Anopheles labranchiae atroparvus*

Isotope	Time of injection (hours pbm)	No mosquitoes used	Mean grain density \bar{x} ± standard error of mean	
			Oviducts	Background
Phenylalanine	24	1	64.20± 1.03	1.55±0.12
	48	1	31.20± 3.19	3.33±0.29
	72	1	78.40± 3.75	3.11±0.19
	96	1	88.60±15.58	1.79±0.89
Tyrosine	24	2	60.11± 5.19	1.72±0.27
	48	1	37.80± 4.33	<1
	72	2	36.60± 1.74	2.83±0.87
DOPA	24	3	6.06± 1.40	1.42±0.33
	48	3	6.86± 1.0	<1
	72	2	3.80± 0.7	<1
	96	1	2.40± 0.60	<1

Each mosquito was pulsed for 30 minutes before being killed.

Mean grain density (\bar{x})
per unit area of oviduct = $\frac{(\text{total number grains counted})}{\text{number mosquitoes used} \times 5 \text{ unit areas}}$

Mean grain density (\bar{x})
per unit area of background = $\frac{(\text{total number grains counted})}{\text{number mosquitoes used} \times 100 \text{ unit areas}}$

1 unit area = 64².

as confirmed by positive Peracetic acid-Alcian blue staining which was successfully blocked by pre-treatment with sodium thioglycolate. Oil red O staining of the oviducts was negative indicating the absence of neutral fat.

It is concluded that the histochemical data of the yolk indicate that it is complex, containing 1:2 glycol groups, protein and neutral lipid. Glycogen is deposited in the follicle apparently after the formation of the egg membranes. The second follicle is characterised by the absence of storage polymers and the yolk complex. The histochemical reactivity of the oviducts is centred chiefly in the cuticular intima. These results are summarised in Table 31.

Table 31 Histochemistry of ovarian follicles and oviducts in *Aedes togoi* and *Anopheles labranchiae atroparvus*

Technique	Current follicle			Penultimate follicle			Oviducts	
	24-48 hours pbm	Oocyte	Yolk	72-96 hours pbm	Oocyte	Yolk	24-96 hours pbm	Intima
	Follicular epithelium	Nurse cells		Egg membranes				
Nucleic acids								
Methyl green pyronin	+++	+	-	-	+	-	+	-
Ribonuclease control	-	-	-	-	-	-	-	-
Carbohydrates								
Best's carmine	-	-	-	-	++	-	+-	-
Amylase control	-	-	-	-	-	-	-	-
Sodium bisulphite toluidine blue	-	-	-	-	+++	-	-	-
Amylase control	+	-	-	-	-	-	-	-
PAS	-	-	+++	-	++	+++	+-	++
Amylase control	-	-	+++	-	-	+++	-	++
Acetylation-PAS	-	-	-	-	-	-	-	-
Acetylation-saponification + PAS	-	-	+++	-	++	+++	-	-
PAS with aldehyde blockade	-	-	-	-	-	-	-	-
AB (2.5)	-	-	-	-	-	-	-	++
AB (1.0)	-	-	-	-	-	-	-	+
LID	-	-	-	-	-	-	-	+-
HID	-	-	-	-	-	-	-	+-
AB (CEC)	-	-	-	-	-	-	-	+-
Proteins								
Bromphenol blue	+++*	+	+++	-	+	+++	+	+
DMAB-nitrite	+	+	+	+	+	+	+	+
Peracetic acid-AB	-	-	-	-	-	-	-	+++
Diazo-coupling	+++*	+	+++	++	-	+++	+	+
Lipids								
Oil red O	-	-	+++	-	-	+++	-	-
Enzymes								
Acid phosphatase	-	-	-	-	-	-	-	-
β -glucosaminidase	-	-	-	-	++	-	-	-
Non specific esterase	-	-	-	-	++	++	-	-

Intense staining = +++, moderate staining = ++, positive staining = +, equivocal staining = +-, negative staining = -. A blank space in the table indicates that no observations of the tissue were made at that particular time interval.

* endochorion only, staining at 48 hours pbm.

DISCUSSION

The current follicle of the newly emerged female mosquito undergoes a limited amount of growth and differentiation prior to the process of vitellogenesis. In an anautogenous species, such as Anopheles labranchiae atroparvus, vitellogenesis will not proceed without a stimulus which is released by the taking of a blood meal.

In an autogenous species there is no resting stage interposed between preliminary follicular growth and differentiation and the succeeding process of vitellogenesis. Laurence (1964) showed that the degree of autogeny of Aedes togoi is dependent upon the size of the adult reared. Laurence and Simpson (1974) showed that large females emerged from the pupa with follicles differentiated 16-40 hours in advance of smaller females. However both large and small females continued to develop until the second day after emergence. After blood feeding the follicular epithelial cells of anautogenous females showed increased mitotic activity coincident with an increase in the number of thymidine-[H₃] marked nuclei, with the total follicular cell number rising during the first 24 hours pbm. The oocyte thus proceeds from Christopher's Stage II to Stage III (Laurence and Simpson, 1974; Laurence, 1975).

In this study Aedes togoi, coming to feed on cats infected with microfilariae of Brugia patei, were aged one week and would have completed the first (autogenous) gonotrophic cycle. The current follicles would be in the resting stage. When injected with uridine-[H₃] at 26.5 hours pbm, all follicular components in the current follicles incorporated the isotope. In individuals injected at 45 hours pbm, the ability of the follicles to incorporate uridine-[H₃] had declined although histochemically, pyronin staining of the follicular epithelium was intense throughout the period 24-48 hours pbm.

In the follicular epithelium, RNA synthesis is likely to be associated with the formation of RNA that would be needed for a succeeding phase of protein synthesis. Ultrastructural studies by Anderson and Spielman (1971) and Mathew and Rai (1975) have shown that the follicular epithelium manufactures and secretes the vitelline membrane and chorion. The marked

ability of the follicular epithelial cells to incorporate uridine-[H₃] during the pre-chorionic phase of vitellogenesis could be related to protein synthesis necessary for the elaboration of the egg membranes. Further studies on the autoradiography of ovarian development in mosquitoes might with advantage incorporate the use of suitable inhibitors of nucleic acid and protein synthesis.

The nurse cells in Aedes togoi and Anopheles labranchiae atroparvus incorporated thymidine-[H₃] and uridine-[H₃] confirming previous observations on these cells (Sharma, Hollingworth and Paschke, 1970; Anderson and Spielman, 1973; Laurence and Simpson, 1974). The nurse cell cytoplasm stains strongly with pyronin. In the meroistic ovaries of higher Diptera the nuclei of the nurse cells produce RNA which is believed to be transported via cytoplasmic bridges to the oocyte (see Huebner, Tobe and Davey, 1975). Freylinger and Roth (1972) suggested that a similar relationship might apply in mosquitoes, although there is no evidence to support this view (Anderson and Spielman, 1973).

In the follicular epithelium and nurse cells of the current follicle of Anopheles labranchiae atroparvus maximum incorporation of glucose-[H₃] and radioactive phenolic amino acids occurred at 24-48 hours pbm, ie, during the vitellogenic and early chorionic phases. An interesting observation was the presence of similar labelled material extracellularly in the haemocoel of Anopheles labranchiae atroparvus during the period 24-48 hours pbm. The material was present in only trace amounts in mosquitoes that had been injected at 72-96 hours pbm and in individuals injected at 24 hours pbm but where the development of the oocyte had not progressed beyond the resting stage. When present the material was aggregated on the surface of organs, principally those in the abdominal cavity. For example, in a female that had been injected with tyrosine-[H₃] at 24 hours pbm the mean grain density of the material sampled was 41.60 ± 4.61 and in a female that had been injected at 48 hours pbm, the mean grain density for the material was in excess of 100. In Aedes aegypti Hagadorn, Fallon and Laufer, 1973 showed that vitellogenin production by the fat body incorporating phenylalanine reached a peak at 28 hours pbm. It is plausible that the phenolic-labelled extracellular material present in the haemocoels of Anopheles labranchiae atroparvus during 24-48 hours pbm represents haemolymph bound vitellogenin precipitated out by the

fixative on to the basement membranes lining the haemocoel. This material was absent in a 24 hour non-parous female where the follicles were still at the resting stage. The label over the fat body was low in comparison to 24 hours blood fed females with normally developing ovaries.

The finding that the follicular epithelium was intensely labelled with DOPA-[H₃] at 24 hours pbm supports the suggestion by Schlaeger and Fuchs (1974c) that DOPA decarboxylase may be synthesised or activated in the follicular epithelium. The presence of both enzyme and substrate in the follicular epithelium would be required for the subsequent manufacture of the chorion. According to Schlaeger and Fuchs (1974a) darkening of the newly laid egg is due to sclerotisation of the chorion and not to melanisation as suggested by Walker and Menzer (1969). Darkening of newly laid eggs does not occur in α MDH injected females. α MDH also prevented darkening of the eggs of injected Anopheles labranchiae atroparvus. For reasons outlined previously in Chapter 4 it is felt here that the darkening of the egg shell could be attributable to either, or a combination of, melanisation and sclerotisation.

The concentration of silver grains in the interface between the surface of the oocyte and the follicular epithelial cells in individuals injected at 24-48 hours with phenylalanine-[H₃] or tyrosine-[H₃] corresponded with the position of the vitelline membrane in routine sections and indicated the importance of phenolic amino acids in the development of egg membranes.

In Anopheles labranchiae atroparvus the ability of the oocyte to incorporate glucose-[H₃] and tyrosine-[H] during the period 24-96 hours pbm was of particular interest since the label seen at 72-96 hours indicated that labelled metabolites were able to traverse the chorion and vitelline membrane. Since some yolk is deposited after micro-pinocytosis ceases and the formation of the egg membranes render the follicle impermeable to small protein molecules, Anderson and Spielman (1971) suggested that the site of yolk synthesis could switch from extra to intra oocytic sites during vitellogenesis. In a later publication Anderson and Spielman (1973) found no evidence to support this idea. The results of this study indicate that their earlier suggestion is worthy of further examination.

In both Aedes togoi and Anopheles labranchiae atroparvus, autoradiography clearly showed that incorporation of radioactive precursors into ripe follicles was negligible except for the oocyte label mentioned above. This low degree of activity suggests that there is little turnover in ripe eggs. Histochemical data tended to support this conclusion. In Aedes togoi fixed at 10-14 days pbm and where the eggs had been retained, the fat body in some individuals was depleted of fat reserves whereas the eggs were still packed with lipid positive yolk spheres. This again indicates that the female does not draw upon the energy reserves locked up in the eggs.

In the penultimate follicle the results showed that growth occurs until the follicle reaches Christopher's Stage II, a process which is coincident with the maturation of the current follicle into the egg. During this previtellogenic period the penultimate follicle showed a more or less uniform incorporation of radioactive nucleosides, glucose-[H₃] and many radioactive amino acids. Histochemical techniques failed to demonstrate the presence of stored glycogen and lipid, although a certain amount of lipid yolk is known to be present. The limited enzyme histochemical data implied that the penultimate follicle is not enzymatically active. In contrast the histochemical data relating to the current follicle showed that within 24 hours pbm the yolk contained substantial stores of neutral mucosubstance and lipid. Glycogen accumulates in the oocyte between 48 and 72 hours pbm and enzymes such as non-specific esterase and β -glucosaminidase were demonstrable. It is possible that the steady uptake of labelled precursors into the penultimate follicle is geared to the process of manufacturing enzymes needed when the follicle is initiated into egg development. Once the follicle has reached a critical level of development and reached the resting stage it is possible to speculate that enzyme production within the follicle is prevented by the inhibiting presence of the current follicle. It is known that the mature follicle prevents development of the penultimate follicle (Else and Judson, 1972; Meola and Lea, 1972b). Once the female has laid eggs enzymes already manufactured but inactive can become activated upon receipt of vitellogenin from the fat body soon after the female had fed on blood. It is interesting that Briegel and Freyvogel (1973) detected differences in non specific esterase patterns in the ovaries of Aedes aegypti during egg development. Fig 9 is a

somewhat tentative scheme of the principal features of ovarian development in mosquitoes. Again the developing filarial larva has to compete for the pool of metabolites during the period of maturation of the ovaries, following the mosquito blood meal, as metabolites are utilised by both the current and the penultimate follicles.

Fig. 9. Tentative scheme of ovarian development in mosquitoes.

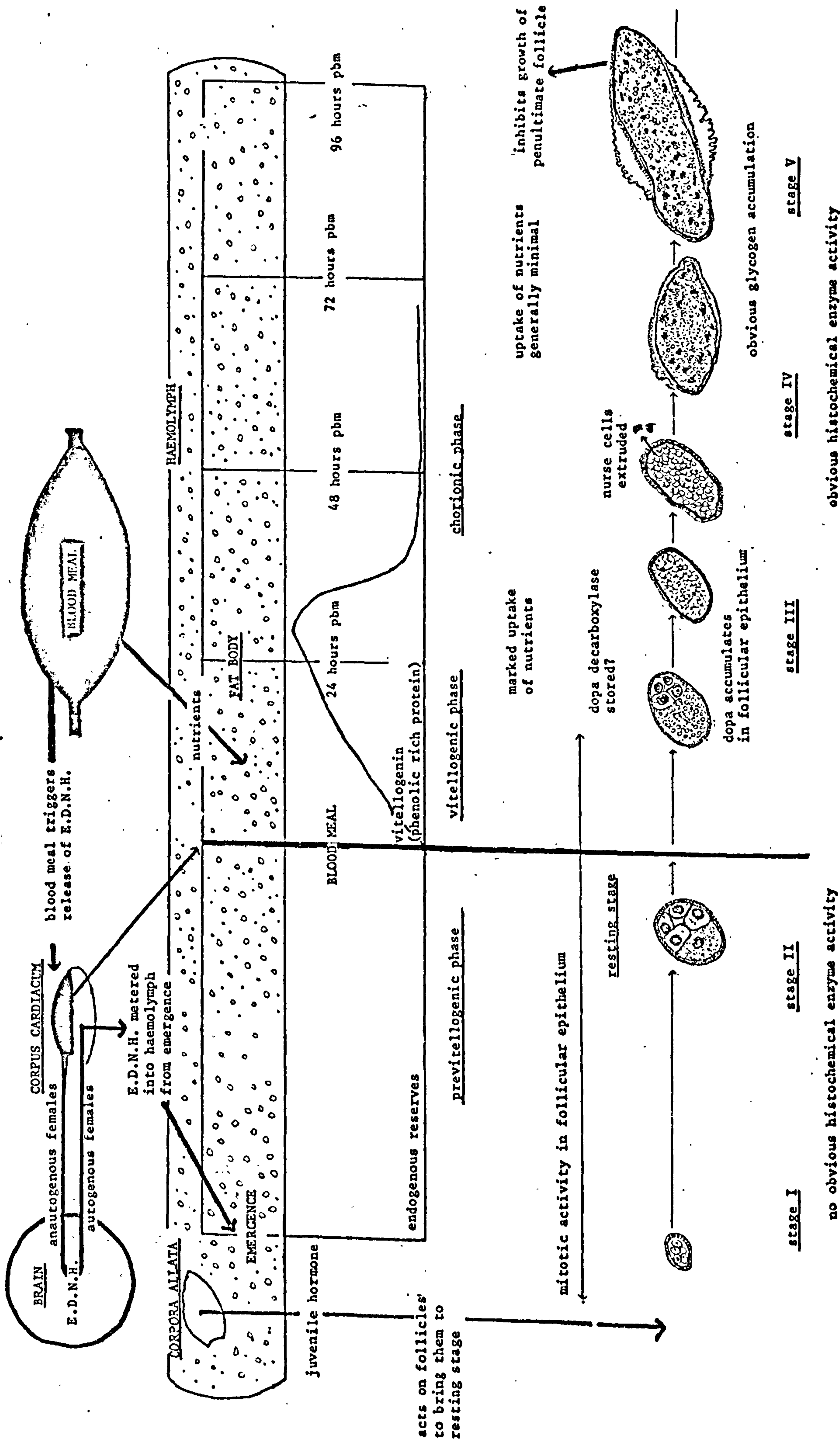


PLATE 19

(a) Incorporation of thymidine-[H₃] into current follicle of Aedes togoi injected at 9 hours pbm. NC = nurse cells, FE = follicular epithelium.

(b) Incorporation of uridine-[H₃] into resting stage follicles of Aedes togoi. FE = follicular epithelium. NC = nurse cells. O = oocyte. All elements of the follicles are labelled. Injected at 5 days pbm. In this individual the eggs have been laid and the penultimate follicles now become the current follicles which are held at the resting stage until the next blood meal.

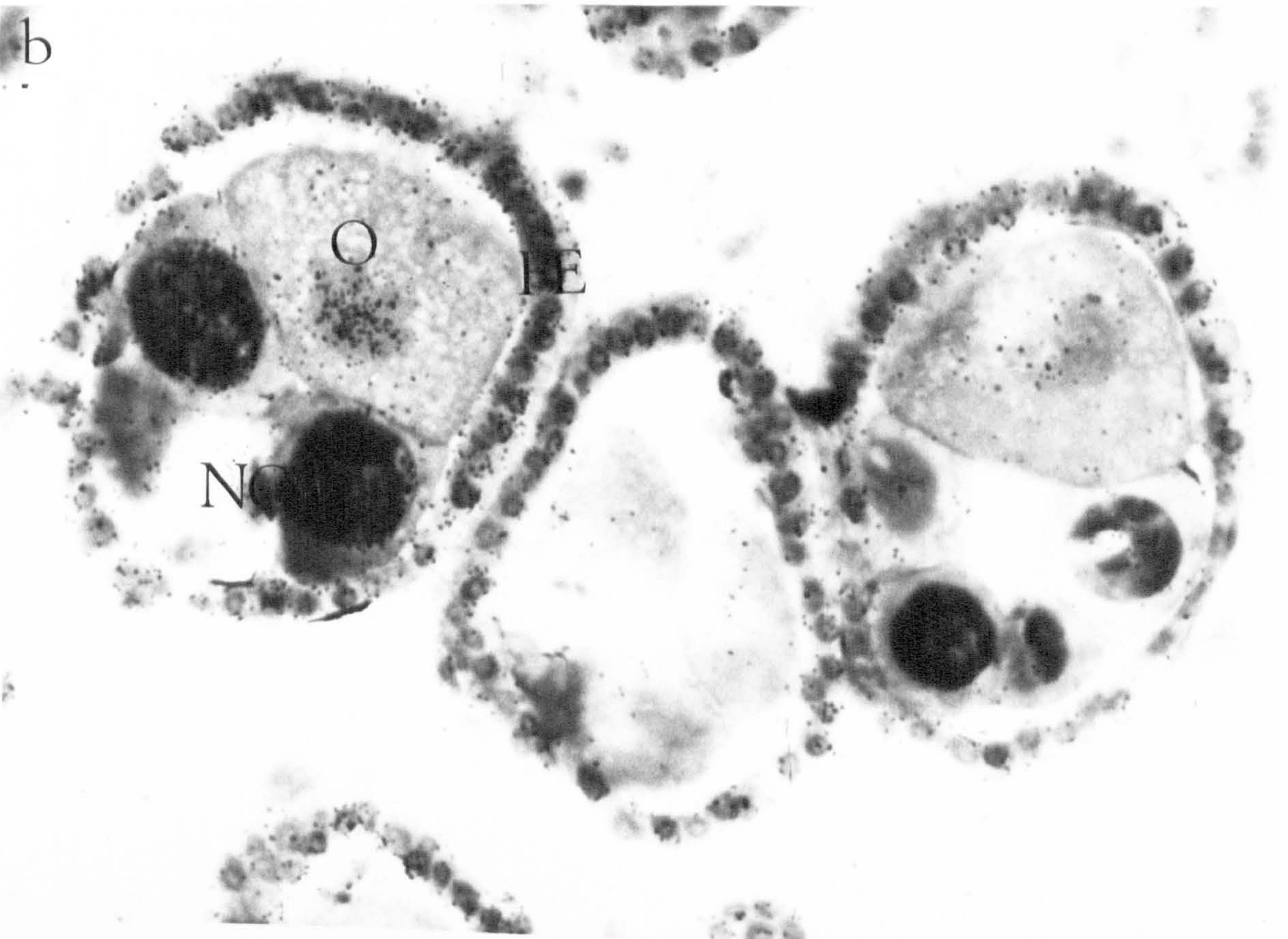
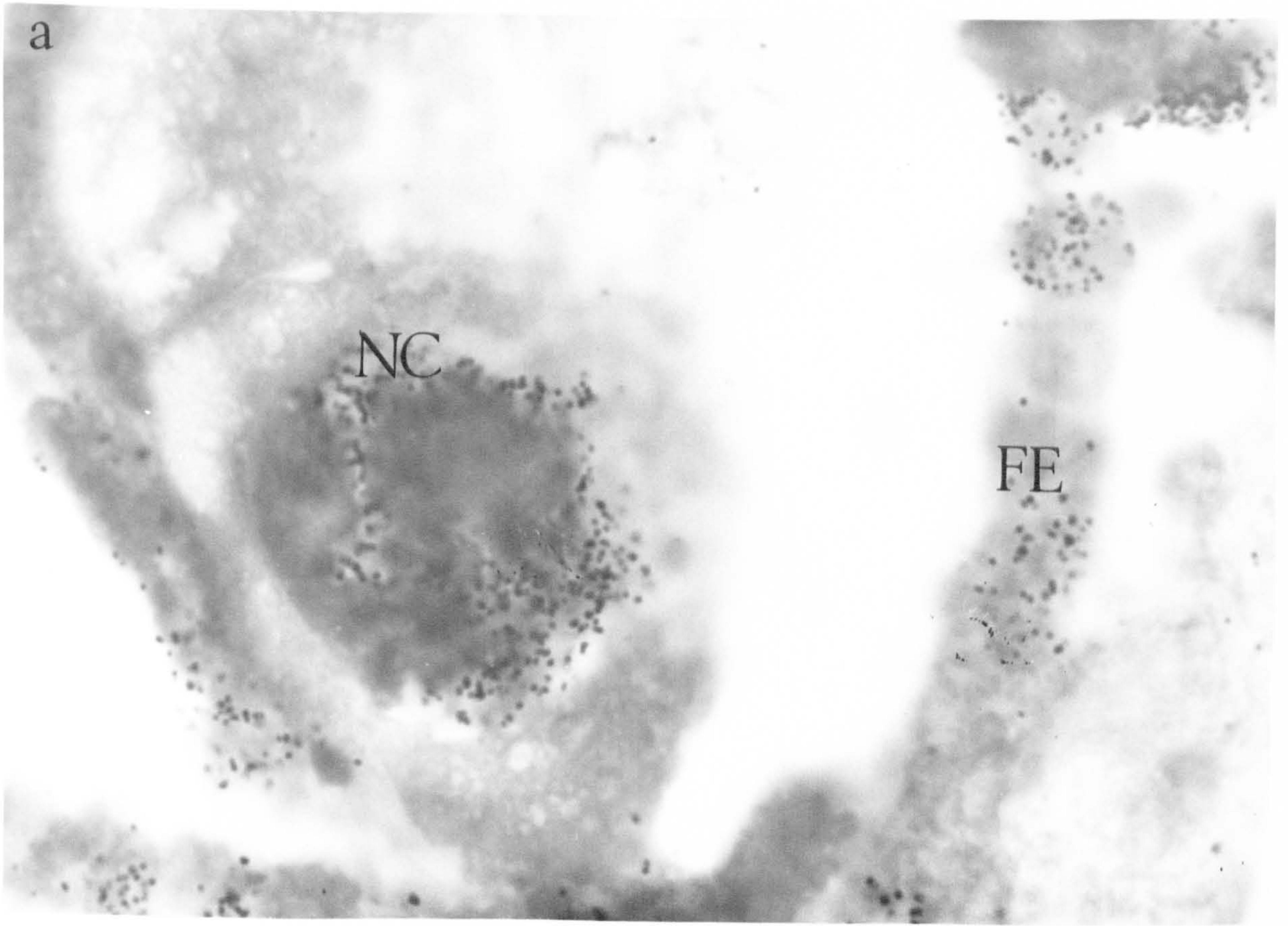
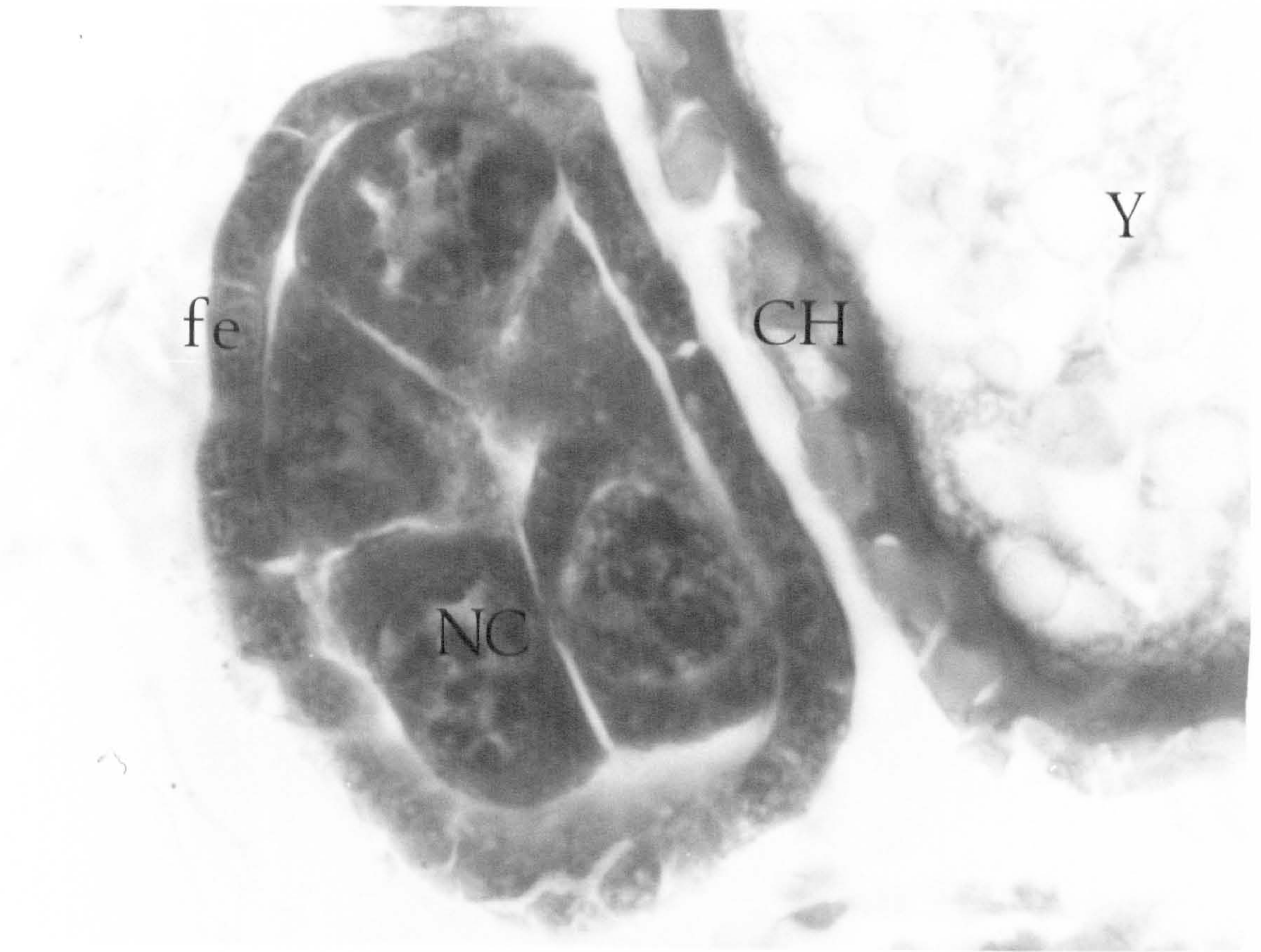


PLATE 20

Incorporation of methionine-[H₃] into penultimate follicle of Aedes togoi. The isotope was injected at 95 hours pbm. fe = follicular epithelium of penultimate follicle. NC = nurse cell. CH = chorion of current egg follicle. Y = yolk spheres. In (a) the focus is on the tissues, in (b) on the silver grains. Note that the label over the penultimate follicles appears to be fairly uniform.

a



b

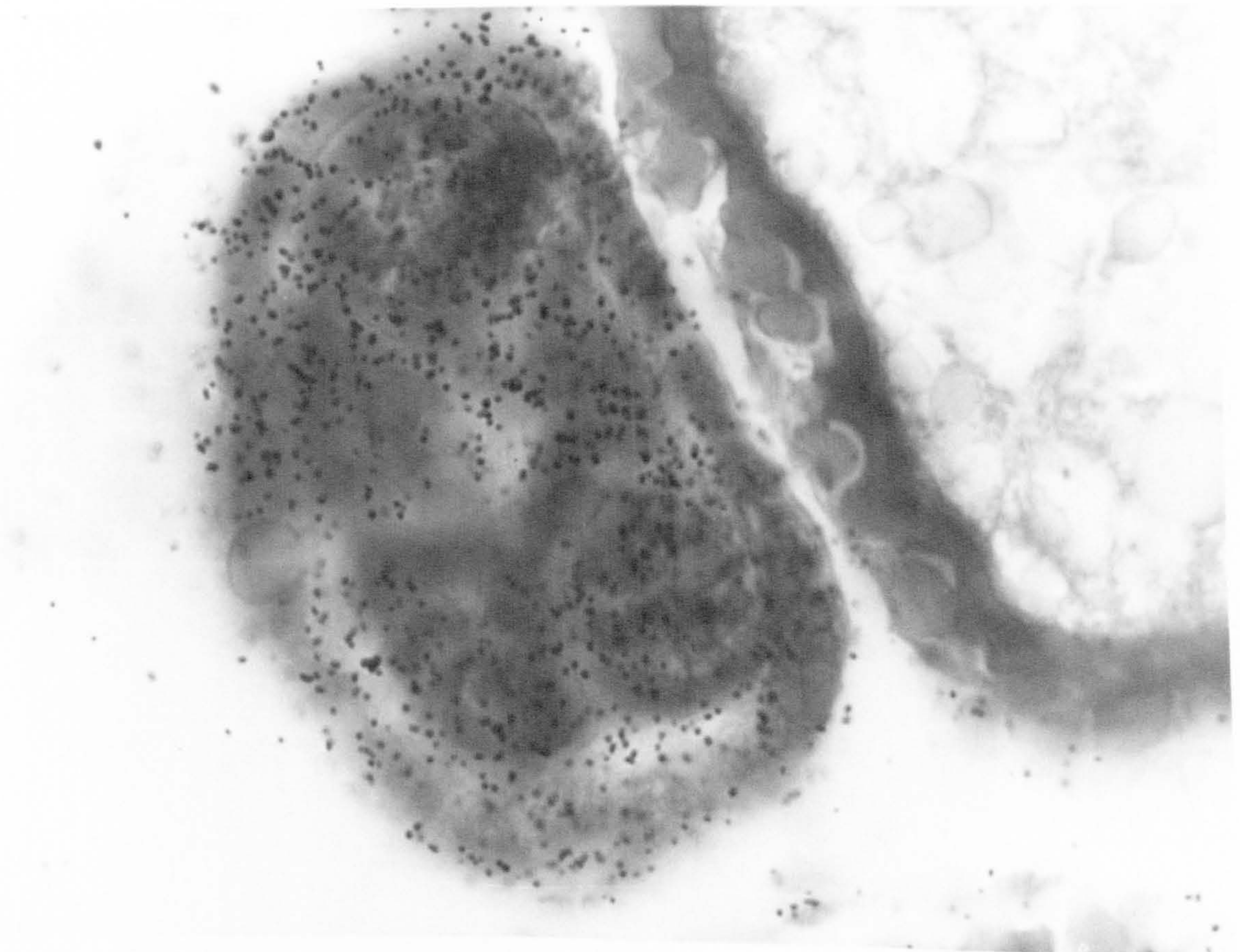


PLATE 21

(a) Ovarian follicles of Anopheles labranchiae atroparvus at 24 hours pbm, fixed in Carnoy and stained with Methyl green pyronin. fe = follicular epithelium of current follicle. NC = nurse cells. Y = yolk spheres.

(b) Ovarian follicles of Anopheles labranchiae atroparvus at 48 hours pbm, fixed in Carnoy and stained with Methyl green pyronin. fe = follicular epithelium. NC = nurse cells. Y = yolk spheres. PF = penultimate follicle.

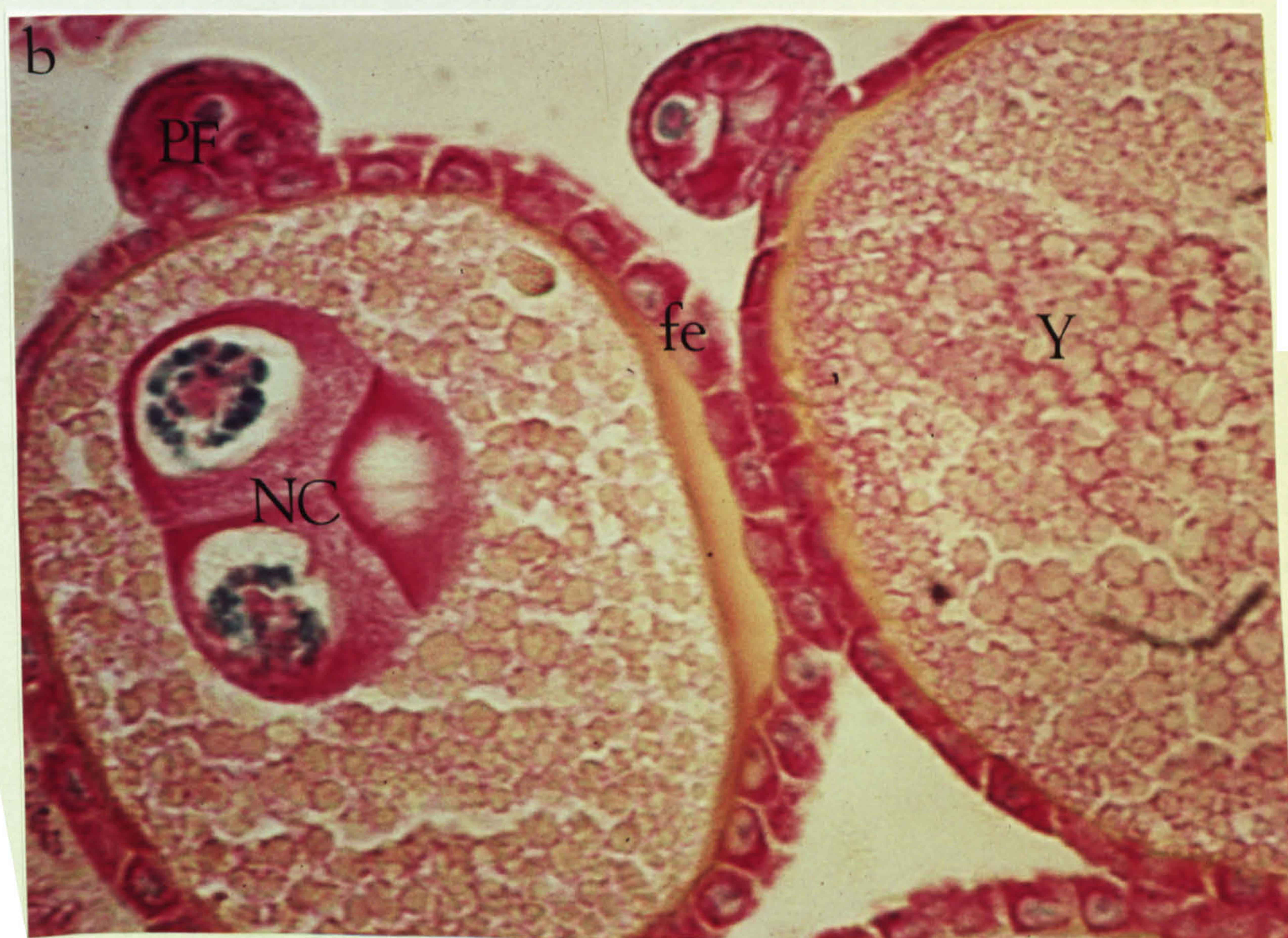
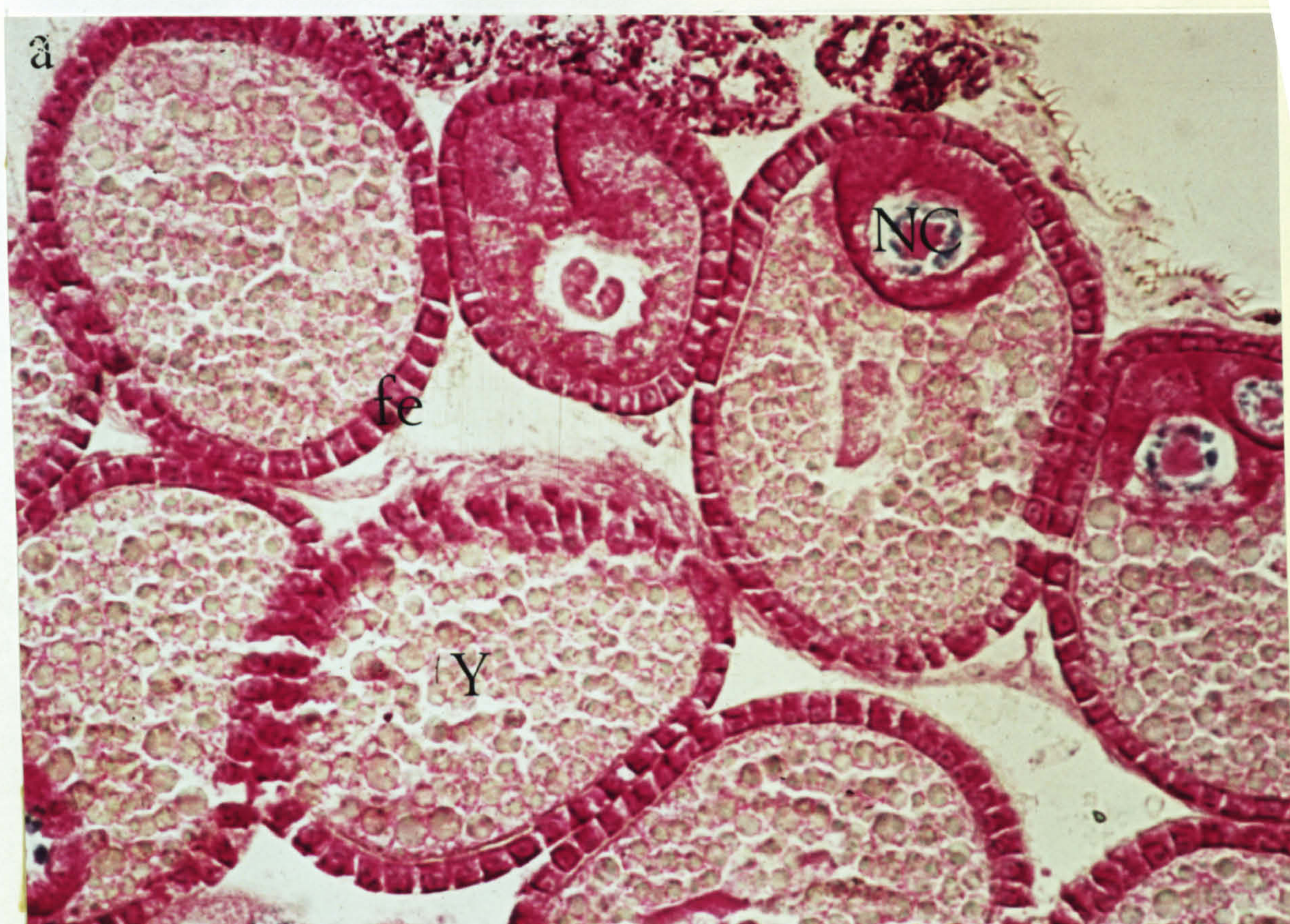


PLATE 22

(a) Ovarian follicles of Anopheles labranchiae atroparvus at 48 hours pbm, fixed in Carnoy and stained with Sodium bisulphite toluidine blue. FE = follicular epithelium of current follicle. NC = nurse cells. Y = yolk spheres. PF = penultimate follicle. fb = fat body.

Note that 48 hours pbm there is no obvious glycogen deposited in the follicles, whereas the fat body adjacent to them is packed with glycogen.

(b) Ovarian follicles of Anopheles labranchiae atroparvus at 72 hours pbm, fixed in Carnoy and stained with Sodium bisulphite toluidine blue. Note the presence of glycogen (g) particles among the yolk spheres. Labelling as for (a) above.

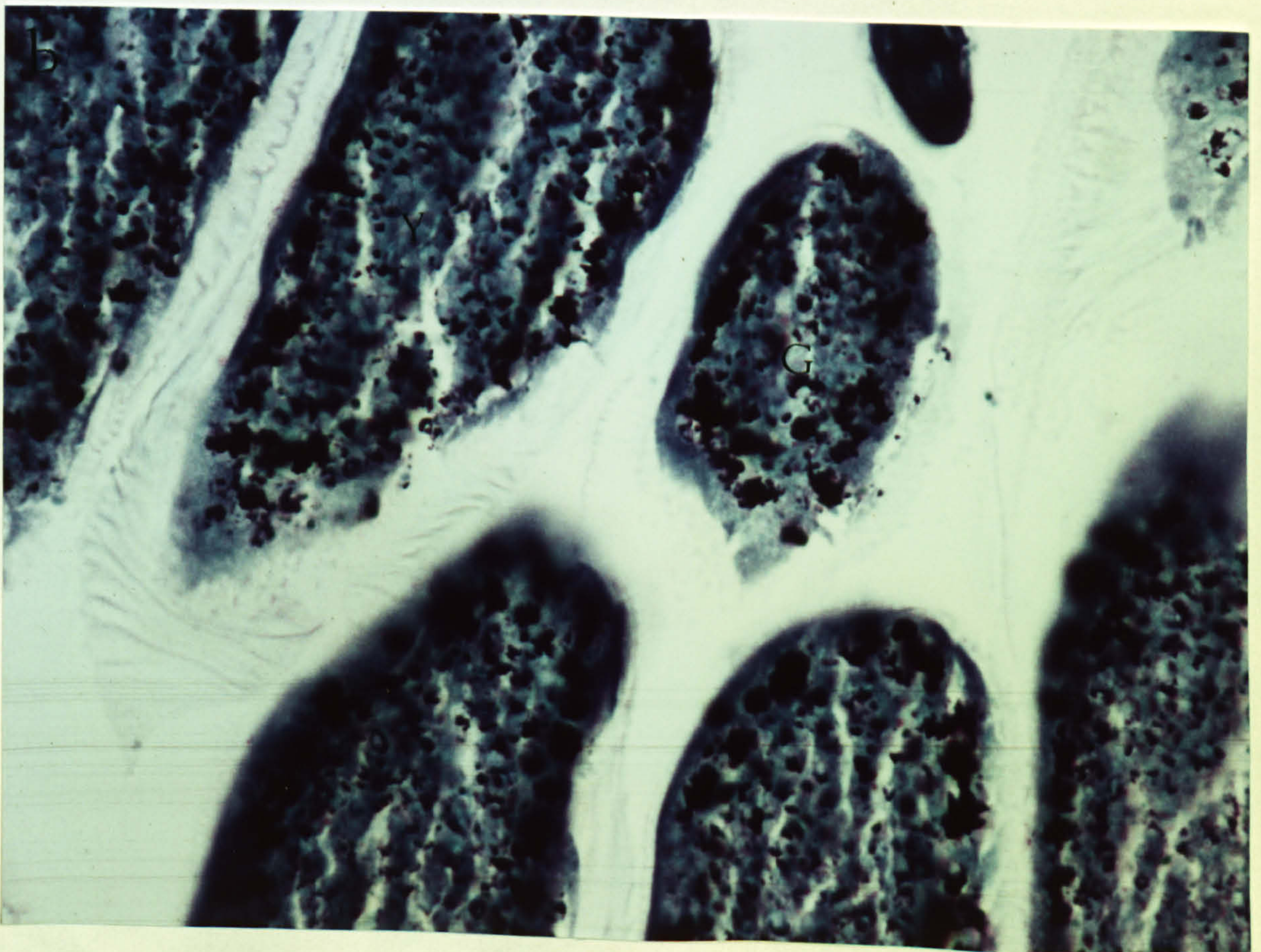
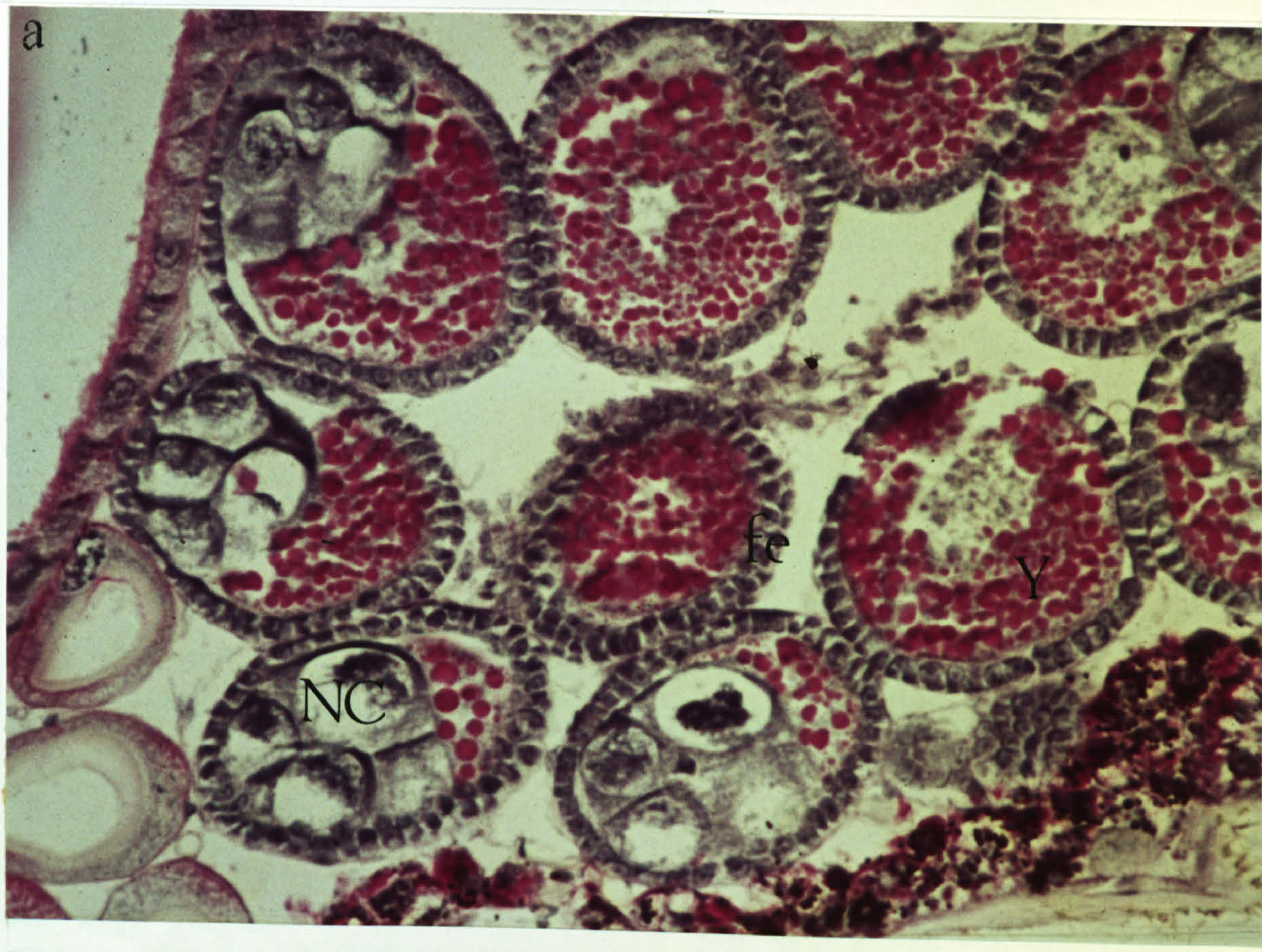


PLATE 23

(a) Ovarian follicles of Aedes togoi at 24 hours pbm, fixed in Carnoy and stained with PAS preceded by amylase digestion. fe = follicular epithelium of current follicle. NC = nurse cells. Y = yolk spheres. Note the strong amylase resistant PAS staining of the yolk spheres.

(b) Ovarian follicles of Aedes togoi at 24 hours pbm, fixed in FA and stained with bromphenol blue. fe = follicular epithelium of current follicles. NC = nurse cells. Y = yolk spheres. Note strong bromphenol blue staining of nurse cell cytoplasm and the yolk spheres.



GENERAL DISCUSSION

Once the female mosquito has fed on blood a number of changes occur mainly in the midgut, fat body and ovaries. In the midgut a peritrophic membrane forms around the contents of the blood meal (Bertram and Bird, 1961; Freyvogel and Jaquet, 1965; Freyvogel and Staubli, 1965; Gander, 1968; Richards and Richards, 1971; Richardson and Romoser, 1972; Romoser, 1971; Romoser and Rothman, 1973; Romoser and Cody, 1975). According to Richardson and Romoser (1972), and Romoser and Cody (1975), the peritrophic membrane is formed in the anterior part of the midgut and is carried posteriorly by peristaltic movement of the gut. In Culex nigripalpis this is completed within 12 hours pbm (Romoser and Cody, 1975), but somewhat later in other species (see review by Gooding, 1972). The ingestion of blood results in the activation of digestive enzymes present in the midgut epithelium and these commence digesting the contents of the blood meal (Gooding, 1972). It is known that proteolytic and lipolytic enzyme activity rises after blood feeding but then declines as the blood meal is digested (Fisk and Shambaugh, 1952; Gooding, 1966; Geering and Freyvogel, 1974; 1975). The latter process is generally completed by 48-96 hours pbm (Gooding, 1972) and this results in an increase of the amino acid concentration of the haemolymph (Thayer, Terzian and Price, 1971). In addition at least some glycogen and fat appear to be derived from the contents of the blood meal as histochemical tests have demonstrated that the abdominal midgut cells of Aedes togoi and Anopheles labranchiae atroparvus accumulate glycogen and neutral fat only at 24-48 hours pbm. Gander (1968) found that the midgut epithelial cells of Aedes aegypti and Anopheles stephensi accumulate carbohydrate and lipid after the blood meal.

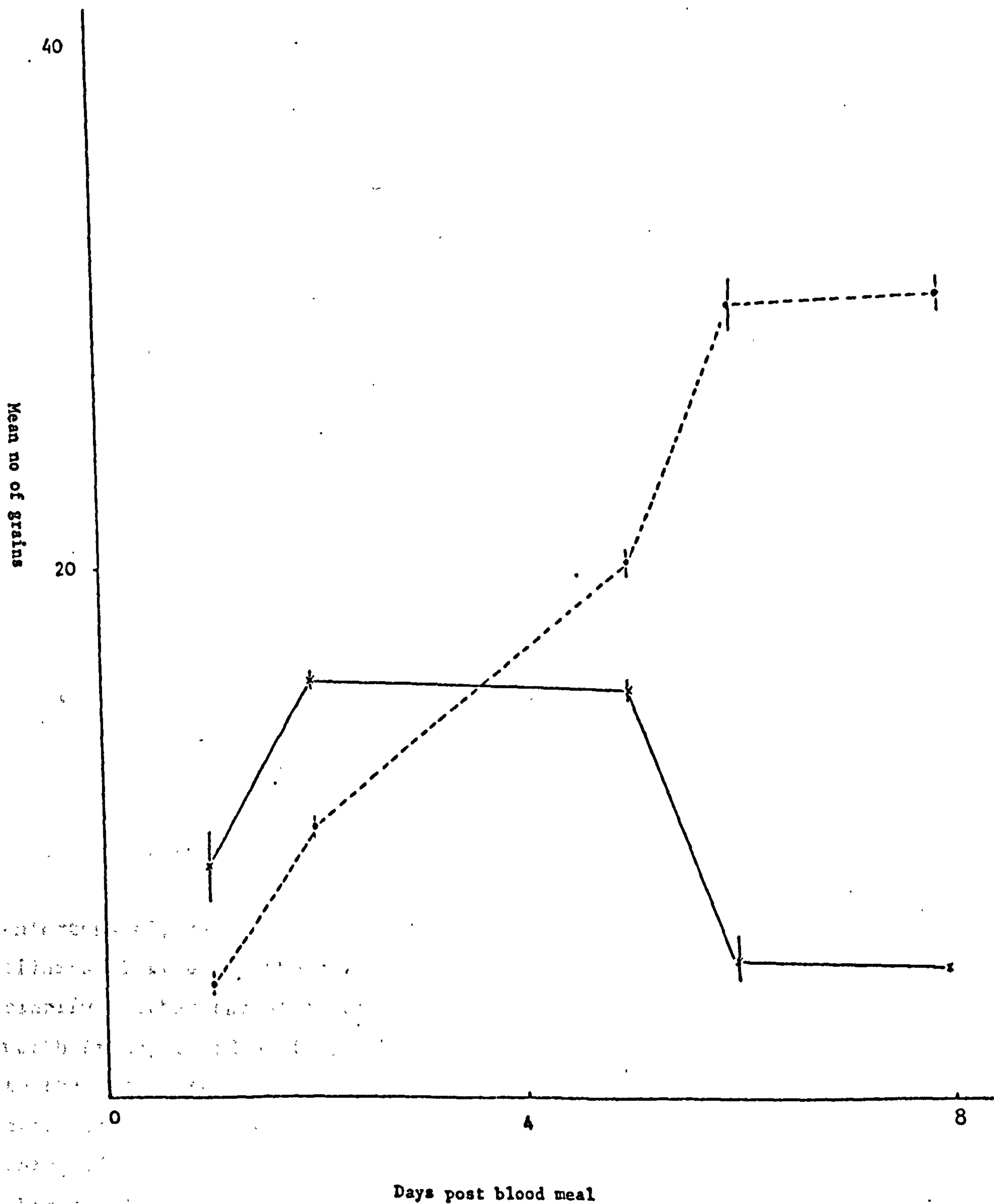
To summarise this activity, during the first 4 days after blood feeding, the midgut epithelial cells have formed a peritrophic membrane, digested the contents of a blood meal and transported the products into the haemolymph. In view of the heavy demands made on these cells, it would be expected that they would require appropriate precursors in readiness for the manufacture of a fresh peritrophic membrane and digestive enzymes at the next blood meal. In this study, using either Aedes togoi and/or Anopheles labranchiae atroparvus it has been shown that the midgut

epithelial cells readily incorporate uridine-[H₃] (Table 19), tritium labelled carbohydrates (Table 20) and amino acids (Tables 21-22) from the haemolymph. This incorporation continues throughout the period 1-4 days pbm.

In many insects the fat body is recognised as a centre for intermediary metabolism and food storage (Kilby, 1963; 1965; Price, 1973), but in mosquitoes the study of the fat body is still in its infancy. In Aedes togoi parasitised by Brugia patei, the autoradiographic evidence in the fat body occurs essentially in the cell nuclei, the label increasing between 1-6 days pbm (Table 15). The histochemical evidence shows that cytoplasmic RNA staining in the fat body is intense during the first 2 days pbm, but is much lower thereafter (Table 19 and Plate 11). In Aedes aegypti total fat body RNA begins to rise at 2 hours pbm, reaches a peak at 12-48 hours pbm and declines to low levels after 28 hours (Hagedorn, Fallon and Laufer, 1973). The above data is consistent with the view that the RNA content of the fat body is relatively high during the period when vitellogenin production is maximal (Hagedorn, Fallon and Laufer, 1973). Thereafter the need for a rich cytoplasmic store of RNA presumably declines. The increased RNA synthesis in the nucleus may represent a preparatory step to the next cycle of rapid cytoplasmic RNA accumulation which will occur when the mosquito takes a further blood meal. In comparison to the fat cell nuclei the developing filarial larvae appear to make less demands on the uridine pool of the host (Fig 10).

Unlike the epithelial cells of the midgut, the fat body of Aedes togoi and Anopheles labranchiae atroparvus incorporated little labelled sugar from the haemolymph but did incorporate a variety of labelled amino acids (Tables 16-18). These amino acids are not converted into storage proteins as histochemical tests demonstrated that the cytoplasm of the fat cells was devoid of discrete protein spheres. Other histochemical tests have shown that the fat body of blood fed females is packed with glycogen and fat. Wigglesworth (1942) showed that the mosquito can convert protein into glycogen and fat (see also Kilby, 1963; 1965; Price, 1973). Consequently, it is possible that some of the labelled amino acids that are incorporated into the fat body are converted to glycogen and neutral fat. Other amino acids are probably used in the manufacture of the yolk protein vitellogenin. The latter is produced by the fat body (Hagedorn and Judson, 1972). Hagedorn, Fallon and Laufer

Fig 10 Relative degree of incorporation of uridine-[H₃] into *Brugia patei* and the fat body of its host *Anopheles labranchiae atroparvus*



-x- = parasite, ----o---- = fat body.
Each point represents the mean of 1 mosquito.
Each bar represents the standard error of the mean.

(1973) found that the synthesis of vitellogenin began at 3-4 hours pbm, reached a peak at 28 hours pbm and declined to low levels at 40 hours. In Anopheles labranchiae atroparvus the incorporation of tyrosine-[H₃] into the fat body decreased steadily over 1-4 days pbm (Fig 2).

Undoubtedly the most pronounced changes that occur in the tissues of blood fed mosquitoes are seen in the ovaries. In the anagenous female mosquito, a blood meal is necessary to release the current egg follicles from a pre-vitellogenic resting stage. After blood feeding the current follicles undergo DNA and RNA synthesis (Sharma, Hollingworth and Puschke, 1970; Freylinger and Roth, 1972; Anderson and Spielman, 1973; Laurence and Simpson, 1974), incorporate vitellogenic yolk protein from the haemolymph (Anderson and Spielman, 1971) and form the egg membranes (Mathew and Rai, 1975). There is also a rise in dopa-decarboxylase activity after the blood meal which is associated with the developing eggs (Schlaeger and Fuchs, 1974a; 1974b; 1974c). The development of the current and penultimate follicle has been followed in Anopheles labranchiae atroparvus and to a lesser extent in Aedes togoi. In the current follicles maximum incorporation of labelled nucleosides, carbohydrate (Table 24) and amino acids (Table 25) occurred during the first 48 hours after blood feeding, whereas in the penultimate follicles incorporation tended to be uniform during the first 4 days pbm (Tables 26, 27).

It is clear from this brief survey that the female mosquito undergoes intense metabolic activity during the first 4 or 5 days after the blood meal. The success or failure of ingested microfilariae to complete development up to the first moult is also determined within this period.

Unfortunately detailed knowledge of fundamental relationships between filarial larvae and their mosquito hosts is lacking. In order to clarify further the state of our ignorance of these relationships, it is worth trying to identify host factors that represent potential barriers to the successful development of the microfilariae into infective third stage larvae. These can be conveniently arranged into a number of categories. Some species of mosquito have well developed cibarial and pharyngeal armatures which can cause mechanical damage to microfilariae as they are ingested (Coluzzi and Trabucchi, 1958; Bryan et al, 1974). Microfilariae may become trapped in the blood meal and fail to penetrate

the wall of the abdominal midgut (Kartman, 1953a; Lavoipierre, 1958; Nelson, 1964; Laurence, 1970) or become lost from the host by defaecation (Kartman, 1953b; see also Boorman, 1960). There is an additional complication for sheathed species of microfilariae. It is important for the latter to undergo exsheathment before migrating through the gut wall. In Brugia malayi, parasitic in Anopheles hyrcanus var. sinensis Weid, Feng (1936) found that the shedding of the sheath within the abdominal midgut was important for normal larval development, as larvae which retained the sheath subsequently developed abnormally. Laurence and Pester (1961) found only a few sheathed microfilariae of Brugia patei outside the stomach of Mansonia uniformis and in this host development of larvae which retained the sheath was abnormal. In Anopheles labranchiae atroparvus microfilariae of Brugia patei exsheath normally.

Once through the wall of the abdominal midgut the microfilariae must be able to migrate successfully to a target site within the host. In mosquitoes the site for filarial development is specific for each filarial genus (Nelson, 1964). Thus, Dirofilaria develops in the Malpighian tubules (Taylor, 1960), Folyella and Breinlia in the fat body (Schacher and Khalil, 1968; Zaman and Chellapah, 1969) and Wuchereria and Brugia in the indirect flight muscles of the thorax (Feng, 1936; Kobayashi, 1940; Laurence and Pester, 1961; Schacher, 1962a; Jayewardene, 1963; Orihel, 1967). The reason for this specificity is unknown but it is apparently important enough to be coded into the behaviour programme of each genus. The direction finding ability of Brugia patei in the refractory host Anopheles labranchiae atroparvus is unimpaired, the majority of the microfilariae that penetrate the gut wall find their way to the flight muscles. In the latter the microfilariae are able to compete successfully for nutrients where the incorporation of precursors into the muscle fibres is low (Tables 6-10).

Once the microfilariae reach the site for development they undergo, in a susceptible host, a series of histologically distinct changes which ensure that each microfilaria is transformed into an infective third-stage larva. This entails initially the division of the G cell and the subsequent modification of the alimentary canal into a functional unit accompanied by changes in the hypodermis and muscle cells (see Beckett and Boothroyd, 1970; Laurence and Simpson, 1971; and Kan and Ho, 1973). The long

slender shape of the microfilaria may be a modification to enable it to live in the capillary network of the vertebrate host (Buckley, 1955). In the intermediate host the initial shortening of the body of the microfilaria into the stumpy sausage form does ensure that the various parts of the alimentary canal are brought into a position which corresponds to that in the infective stage larvae (Feng, 1936; Laurence and Simpson, 1971). In susceptible hosts this is achieved by an ordered sequence of growth and cell divisions. In refractory hosts integrated growth and cell division does not occur (Laurence, 1970; Oothuman, Simpson and Laurence, 1974) and such larvae will experience difficulty in progressing beyond the sausage form. The latter may well represent a threshold to further development. Yoeli, Alger and Most (1958) found that when microfilariae of Dirofilaria immitis were introduced experimentally into larvae of the Wax Moth Galleria mellonella, division of the G cell occurred and that the microfilariae succeeded in shortening into sausage forms. However, further development did not occur in the majority of the experimentally introduced larvae. In Brugia the sausage form is reached in less than 48 hours after entry (Feng, 1936; Esslinger, 1962; Schacher, 1962; Laurence and Pester, 1961; Jayawardene, 1963; Orihel, 1967; Laurence and Simpson, 1971).

It is not known whether the uncoordinated and eventually abortive development of Brugia patei in Anopheles labranchiae atroparvus is attributable to single or multiple factors. Obvious ones to consider here are whether there is any impairment of either DNA or RNA synthesis and/or a failure to incorporate amino acids needed for protein synthesis. The autoradiographic data suggests that larval Brugia patei may be entirely independent of an external source of pyrimidines necessary for DNA synthesis. Both normal and abnormal larvae failed to incorporate the DNA marker thymidine-[H₃] at any of the three larval stages of development although nuclei in the midgut, fat body and ovarian follicles of the host mosquitoes readily incorporated the isotope. Omar and Gwadz (1974) failed to label with thymidine-[H₃] the nuclei of Brugia pahangi developing in Aedes aegypti and also showed that the larvae incorporated little adenine-[H₃]. In this study it has been established that both normal and abnormally developing larvae of Brugia patei incorporated the pyrimidine nucleoside uridine-[H₃]. Uptake was intense in sausage forms at 45 hours pbm compared to that recorded for infective larvae (Table 1). Taylor (1960)

has commented that the sausage stage of the filarial larva has an optimum surface to body ratio. However there is little doubt of the absorptive capacity also of older first stage larvae. Both normal and abnormally developing larvae of Brugia patei incorporated the amino acids histidine-[H₃], proline-[H₃], arginine-[H₃], lysine-[H₃], methionine-[H₃], cystine-[H₃], iso-leucine-[H₃], leucine-[H₃], valine-[H₃], phenylalanine-[H₃], tyrosine-[H₃], and tryptophan-[H₃]. Infective-stage larvae also incorporated leucine-[H₃] at 11 days pbm as did abnormal larvae of the same age (Tables 4, 5). Minor differences of uptake between normal and abnormal larvae possibly reflects differences in labelling pattern between the host mosquitoes. However the data is sufficiently clear to rule against a simple nutritional explanation for the failure of Brugia patei to develop normally in Anopheles labranchiae atroparvus.

In a refractory mosquito host a major barrier to successful filarial development is the onset of a host reaction against the larvae. In Anopheles labranchiae atroparvus development of Brugia patei appears morphologically normal for approximately the first 24 hours after entry, but thereafter further development becomes increasingly abnormal. Many of the larvae become encapsulated by a substance which has been shown histochemically to contain neutral mucosubstance and what appears to be melanin pigment (Table 12). Autoradiographic data has shown that the encapsulation substance incorporated phenylalanine-[H₃], tyrosine-[H₃], DOPA-[H₃], arginine-[H₃], glucose-[H₃], and galactose-[H₃]. The phenolic acids are known melanin intermediates and these were also incorporated by pigment associated with integumentary wounds and in the mosquito integument itself (Table 11). The monosaccharides were presumably incorporated into the mucosubstance component of the host reaction. The encapsulation substance was labelled in mosquitoes injected at 24-96 hours pbm. The presence of this material around the larvae did not prevent uptake of labelled uridine-[H₃] and the tritium labelled amino acids, so abnormal larval development is unlikely to be due to a simple mechanical obstruction of all the absorptive sites of the body surface. However the signal that initiates abnormal development may coincide with the earliest deposition of encapsulation material onto key sites at the surface of the larvae. The frequent finding in larvae of melanised material located only over the excretory and anal vesicles, particularly at 24-48 hours after entry, does suggest that these areas are the focus for the initial deposition of

encapsulation material. In nematodes the body wall consists of an outer cuticle which lies over an inner hypodermis (see reviews by Lee, 1966; 1972; Bird and Bird, 1969; Bird, 1971). The hypodermis is believed to be the site for the manufacture and secretion of cuticular protein (Kan and Davey, 1968; Samoiloff and Pasternak, 1969; Johnson, Van Gundy and Thomson, 1970; Lee, 1970; Bonner and Weinstein, 1972; Minier and Bonner, 1975). In this study the body wall of both normal and abnormal larvae of Brugia patei incorporated many different tritium-labelled amino acids. Even so the hypodermal and muscle cells of abnormal larvae do not grow sufficiently and abnormal larvae were stunted in appearance (Oothuman, Simpson and Laurence, 1974). These workers also observed that some larvae of Brugia patei parasitic in Anopheles labranchiae atroparvus were able to manufacture a new cuticle in preparation for the moult to the second larval stage, but that ecdysis did not occur. This observation, together with the disorganised pattern of growth of the intestine of these larvae, suggests a failure in the mechanisms which coordinate cellular development.

In insects larval development, including moulting, is under hormonal control (Fraenkel and Hsiao, 1965; Fogal and Fraenkel, 1969; Mills and Whitehead, 1970; Wigglesworth, 1964; 1970b; Post, 1972; Seligman and Doy, 1972; Vandenburg and Mills, 1974; 1975). It is possible that abnormalities in filarial development in refractory mosquitoes may arise because of a malfunctioning of the neuroendocrine system of the parasite or alternatively to the inability of the parasite to utilise or manipulate the endocrine system of the host (see reviews by Davey and Hominick, 1973; Riddiford, 1975). However evidence for the existence of a neuroendocrine system in larval filarial nematodes is limited to reports of the presence of electron dense granules in ganglionic nerve cells of microfilariae which are similar in appearance to neurosecretory granules (Kozek, 1971; McLaren, 1972; O'Leary, Bemrick and Johnson, 1973; Laurence and Simpson, 1974). Furthermore, present evidence indicates that filarial development in mosquitoes is independent of the hormones which regulate egg development (Yoeli, Upmanis and Most, 1962; Terwedow and Rodríguez, 1973; Townson, 1974; Gwadz and Spielman, 1974). These latter workers studied the effects of decapitation or ablation of the median neurosecretory cells or corpora allata on the development of Brugia pahangi in susceptible and refractory strains of Aedes aegypti. The parasite developed normally in the

decapitated and ablated females of the susceptible strain and still failed to develop in females of the refractory strain. The larvae also developed in unfed females and in males, and in females that had received topical applications of ecdysone or juvenile hormone. Similarly, injected ecdysone or juvenile hormone had no effect on filarial development. In contrast, in the nematode Nematospiroides dugius, Dennis (1976) has shown that exogenous α -ecdysone stimulated the final moult from larva to adult whereas juvenile hormone inhibited this process.

This study has concentrated on the early period of filarial development in normal and refractory mosquito hosts. During this period the susceptible host Aedes togoi appears to be unaffected by the presence of Brugia patei. In the refractory host Anopheles labranchiae atroparvus, the female mosquito can mount a successful host reaction against Brugia patei and still mature the first batch of eggs that result from the taking of the infected blood meal. This is so despite the fact that phenolic substrates are required for encapsulation of the parasite, and for hardening and darkening of the egg membranes. It is possible that the female mosquito has a large reserve pool of these substrates. It is interesting that uninfected female Anopheles labranchiae atroparvus incorporates higher levels of DOPA-[H₃] than females infected with Brugia patei. This area of the study deserves following up in some depth. In fact, a study of phenol metabolism in the mosquito is needed in order to exploit fully the potential of insecticides which apparently interfere with phenol metabolism (see Busvine, 1972).

In the susceptible host, late second stage larvae of Brugia can feed on muscle fragments derived from the host (Beckett and Boothroyd, 1970; Kan and Ho, 1973), and this stage and the infective stage have been shown to incorporate labelled amino acids from the haemolymph. It is possible that during this final phase of development in the mosquito host, the parasite may have a debilitating effect on the mosquito, and may be more comparable than the first stage larvae to mermithid parasitisation of insects (see Gordon, Webster and Mead, 1971; Gordon, Webster and Hislop, 1973). Mature filarial larvae damage the flight muscles when leaving them to enter the haemocoel (Beckett, 1971). This may explain the reduced flight capacity of filarially infected mosquitoes (Townsen, 1970; Hockmeyer et al, 1975). Alternatively more mature filarial larvae

may be a drain on the depleted reserves of the female, affecting subsequent gonadotrophic cycles (Javadian and MacDonald, 1974). The application of ARG techniques to study mature filarial infections in the intermediate host may help to resolve these problems.

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